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PRINCIPAL INVESTIGATOR: Gregory I. Liou, PhD

CONTRACTING ORGANIZATION: GEORGIA HEALTH SCIENCES UNIVERSITY

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Gregory I. Liou, PhD; Saif Ahmad, PhD; Ahmed Elsherbini

E-Mail: giliou@gru.edu; **saabdualazez@kau.edu.sa**; aelsherbini@gru.edu

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14. ABSTRACT Traumatic optic neuropathy (TON) is a type of injury commonly seen in the war. There are currently no proven treatments that reverse the damage in TON. The proposed mechanism of TON involves optic nerve injury-induced activation of retinal microglial cells and their release of pro-inflammatory cytokines, and retinal ganglion cell (RGC) death. As a self-defense system, activated microglial cells also release adenosine, which attenuates inflammation via adenosine receptors (AR)s, including A2AAR. Although AR agonists attenuate inflammation, the way to minimize nonspecific effects associated with systemic administration of these agonists remains unclear. Released adenosine levels in the injured brain are mainly regulated by adenosine kinase (AK). Inhibition of AK potentiates local extracellular adenosine levels at cell and tissue sites which are undergoing accelerated adenosine release. Thus, AK inhibition represents a mechanism to selectively enhance the endogenous protective actions of adenosine during cellular stress. Our studies have shown that microglial activation, retinal inflammation, and RGC death occur in the mouse model of TON, and that A2AAR signaling provides protection from TON. Further, our preliminary data suggest that AK inhibitor (AKI)-enhanced A2AAR signaling provides protection from TON in mice. Therefore, inhibition of AK potentially amplifies the therapeutic effects of site- and event-specific accumulation of extracellular adenosine, which is of highly translational impact.

15. SUBJECT TERMS
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INTRODUCTION

Traumatic optic nerve injury is commonly seen in motor vehicle accidents, assaults, and in the theater of war. Traumatic optic nerve injury is usually the consequence of a severe blunt head trauma, often a frontal blow severe enough to cause loss of consciousness. Unfortunately, there are currently no proven treatments that can prevent the damage associated with an acute traumatic optic neuropathy (TON). Prognosis for the recovery of vision in TON is still poor, nevertheless, animal models for TON are often used, mostly because they are easy to perform and can be well standardized [1]. Retinal ganglion cell (RGC) death is known to be a fundamental pathological process in traumatic optic injury including TON. Several common mechanisms have been hypothesized to underlie apoptotic processes, including interruption of trophic support, oxidative stress, and increased extracellular glutamate levels that result in excitotoxicity. These stimuli associated with the injured RGCs often activate retinal microglia, which release pro-inflammatory cytokines and cytotoxic molecules to further exacerbate the degenerative process [2]. These findings suggest that pharmacological interventions that reduce inflammation may be effective neuroprotectants for TON.

Adenosine is centrally involved in the signaling cascade of related events, including anti-inflammatory actions, angiogenesis, oxygen supply/demand ratio, and ischemic pre- and post-conditioning [3]. Under these circumstances, the local levels of extracellular adenosine are increased due to the increased need for energy supplied by ATP [4]. The increased extracellular adenosine at inflamed sites can protect against cellular damage by activating the A_{2A} adenosine receptor (A_{2A}AR), a Gs-coupled receptor [5]. Extracellular adenosine re-uptake by the equilibrative and concentrative nucleoside transporters (ENT and CNT) allows for adenosine conversion to AMP by adenosine kinase (AK) [6], decreases extracellular adenosine levels, and terminates the protective effect of A_{2A}AR. The removal of extracellular adenosine is predominantly regulated by AK via conversion of adenosine into AMP. The extracellular levels of adenosine are largely dependent on the intracellular activity of AK whereas the degradation of adenosine into inosine by adenosine deaminase (ADA) plays only a minor role in regulating adenosinergic function [7, 8].

It was reported that the degree of brain injury directly depends on expression levels of AK and the resulting extracellular levels of adenosine [8]. Indeed, transgenic mice overexpressing AK are highly susceptible to stroke-induced brain injury [9]. We have studied the role of A_{2A}AR in diabetic retinopathy [5] and in TON [10]. We have also evaluated AK in regulating adenosine signaling in diabetic retinopathy [11], and aim to evaluate the role of AK in TON. We hypothesize that adenosine kinase inhibitors (AKI) could play the same protective role in the TON-injured retina.

BODY

A. Statement of work

We seek to understand the mechanism of inflammation in TON in an effort to control RGC death.

Task 1: To test the hypothesis that A_{2A}AR-cAMP signaling is anti-inflammatory in TON.
Task 2: To test the hypothesis that anti-inflammation by A_{2A}AR-cAMP signaling is impaired in TON.
Task 3: To test the hypothesis that enhancing A_{2A}AR-cAMP signaling attenuates inflammation in TON.

B. Hypotheses to be tested

Hypothesis 1. We hypothesize that a mechanism of anti-inflammation mediated by adenosine receptor A_{2A} (A_{2A}AR) signaling exists in retinal microglial cells. In the setting of TON, however, this process is

overwhelmed by the pro-inflammatory state. We further hypothesize that a selective $A_{2A}AR$ agonist effective in reducing inflammation in other disease processes is of utility in TON.

Hypothesis 2. We hypothesize that an imbalance in adenosine formation and metabolism in the retinal microglia may contribute to retinal complications in the setting of TON.

The above tasks have been performed, hypotheses 1 and 2 have been tested and the results have been published [10]. The article including Abstract, Introduction, Experimental Design, Results, Discussion and References are attached as an Appendix. The remaining progress reports are specifically for hypothesis 3:

Hypothesis 3. We have identified an imbalance in adenosine formation and metabolism in the retinal microglia participated by AK contribute significantly to retinal complications in diabetic retinopathy [11]. We hypothesize that AK may contribute similarly to retinal complications in the setting of TON.

C. Experimental Design and Results

Experimental Design

Preparation of AKI

A selective AKI, 4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl)pyrido[2,3-dipyrimidine (ABT-702, 5 mg) from Santa Cruz was dissolved in 0.25 mL of DMSO (20 mg/mL) and then in 9.75 mL of distilled water to prepare a 0.5 mg/mL stock solution. The solutions were aliquoted and stored at −20 °C for later use. An equivalent volume of vehicle solution was administered to the control animals. ABT-702 was used previously to study the effect of AK inhibition on neuronal inflammation [12] and age-related hearing loss [13]. ABT-702 was 1300- to 7700-fold selective for AK compared with a number of other neurotransmitter and peptide receptors, ion channel proteins, neurotransmitter/nucleoside reuptake sites, and enzymes, including cycloxygenases-1 and -2 [14] .

Animal Preparation and Experimental Design

All procedures with animals were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare publication, NIH 80-23) and Georgia Health Sciences University guidelines. Eight-week-old male mice in C57BL/6 background, were matched according to sex, age, and weight. Mice were anesthetized according to standard protocol and limbal conjunctival peritomy was performed on one eye of each mouse. Forceps dissection under the conjunctiva posteriorly allowed access to the optic nerve, upon which pressure was placed 1mm posterior to the globe until pupillary dilation was noted (approximately 10 seconds). Blood vessel close to optic nerve was carefully avoided in TON surgery. Mock-operated contralateral eye served as the control. After one week, all mice were sacrificed. Eyes were enucleated and sectioned for immunohistochemistry. Retinas were harvested for Western or Real Time PCR analysis. In pharmacologic studies, mice were rendered optic nerve crush and then injected I.P with vehicle (DMSO), or ABT-702 twice a week [15].

Primary retinal microglia culture

Microglial cells were isolated from retinas of newborn Sprague Dawley (SD) rats according to a previous procedure [16] with minor modifications. Briefly, retinas were collected into phosphate-buffered saline and digested with 0.125% trypsin for 3–5 min before mixing with Dulbecco's Modified Eagle Medium (DMEM)/ F12 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Retina pieces were then filtered through a mesh (100 μm), collected by centrifugation, resuspended in culture medium and plated onto T75 cell culture flasks (Corning, NY) at a density of 2×10^5 cells/cm^2. After 2 weeks, microglial cells were harvested by shaking the flasks at 100 rpm for 1 h. Immunocytochemical studies showed that more than 95% cultured cells stained positively for Iba1. Almost none of these cells showed positive staining for GFAP, indicating that majority of the isolated cells were microglia and were not contaminated with astrocytes or Müller cells (data not shown).

Drug treatment effects on cultured microglial cells
Microglial cells were seeded at a density of 5×10^5 cells/well in a collegen-1-pretreated 24-well tissue culture plate. One day after seeding, the cultured wells were washed with Cellgro Complete (Mediatech, Manassas, VA) and incubated in the same media with various treatments. Cells were pretreated with AR antagonists (all are from Sigma-Aldrich except ZM 241385, which is from Tocris) at the indicated concentrations for 30 minutes at 37°C, followed with ABT-702 or vehicle for 30 minutes at 37°C. Microglial activation was then achieved by addition of Amadori-glycated albumin (AGA; Sigma) with undetectable endotoxin (<0.125 units/mL, 10 EU = 1 ng lipopolysaccharide; Lonza, Basel, Switzerland) to each well at a final concentration of 250 or 500 μg/mL at indicated time points [5, 17]. After the indicated time course, culture media were collected and assayed for TNF-α by ELISA.

ELISA for TNF-α
TNF-α levels in the supernatants of culture media were estimated with ELISA kits (R & D, Minneapolis, MN) per the manufacturer's instructions. Standards and samples were added and bound by the immobilized antibody. After washing an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells followed by a substrate solution yielding a colored product. The intensity of the color was measured at 450 nm. The sample levels were calculated from the standard curve and corrected for protein concentration.

Quantitative Real Time-PCR
Total RNA was isolated from mouse retina using SV Total RNA Isolation kit (Promega, Madison, WI) following manufacturer's instructions, and the quality of the RNA preparations was monitored by absorbance at 260 and 280 nm (Helios-Gamma, Thermo Spectronic, Rochester, NY). The RNA was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using iScript reagents from Bio-Rad on a programmable thermal cycler (PCR-Sprint, Thermo Electron, Milford, MA). Fifty ng of cDNA was amplified in each qRT-PCR using a Bio-Rad iCycler, ABgene reagents (Fisher scientific) and appropriate primers (**Table 2**). Average of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA was used as the internal control for normalization.

Western blot analysis
Dissected individual mouse retinas were homogenized in modified RIPA buffer (Upstate, Lake Placid, NY), containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% deoxycholate, supplemented with 40 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride and 1:100 (v/v) of proteinase inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation at 12,000 ×g at 4°C for 30 minutes. Protein was determined by DC Protein Assay (Bio-Rad, Hercules, CA) and 100 μg was boiled in Laemmli sample buffer, separated by SDS-PAGE on a gradient gel (4 to 20%) (Pierce, Rockford, IL), transferred to nitrocellulose membrane and incubated with specific antibodies. Antibodies for β-actin, ICAM-1, ENT1, A_{2A}AR and AK (Santa Cruz Biotechnology, Santa Cruz, CA) were detected with a horseradish peroxidase-conjugated antibody and enhanced chemiluminescence (ECL) (Amersham BioSciences, Buckinghamshire, UK). The same filter was re-probed with control antibodies, such as those for the actin. Intensity of immunoreactivity was measured by densitometry.

Immunolocalization studies Immunofluorescence analysis was performed using frozen eye sections. Briefly, cryostat sections (7μm) were fixed in 4% paraformaldehyde, blocked with Dako protein block serum-free and then incubated overnight at 4°C with primary antibodies: rabbit anti-Iba-1 (Proteintech Group), or goat anti-ICAM-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-AK (Santa Cruz Biotechnology, Santa Cruz, CA). Thereafter, sections were briefly washed with PBS and incubated with appropriate secondary antibodies. Slides were examined by fluorescent microscope. Specificity of the reaction was confirmed by omitting the primary antibody, or by using non-immune IgG.

Immunohistochemistry of cleaved, activated caspase-3 was performed as follows. Retinas frozen sections were fixed in 4% paraformaldehyde, rinsed in PBS, blocked with 0.3% H_2O_2 then Mouse on Mouse (M.O.M.) Immunoglobulin Blocking Solution (Vector Laboratories, Burlingame, CA), and reacted with antibodies

detecting cleaved, activated caspase-3 (Cell Signaling Technology) for 16 to 20 hours at room temperature. Sections were washed, and reacted with M.O.M. biotinylated anti-mouse Ig reagent (1:250), followed by M.O.M. ABC reagent. Color was developed with 3,3′-diaminobenzidine (DAB) as substrate.

Terminal dUTP nick end-labeling (TUNEL)

TUNEL was performed in frozen sections using the TACS-2 TdT Fluorescein In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD) counter-stained with propidium iodide, according to the manufacturer's suggestions. Briefly, sections were hydrated with Alcohol 100%, 95%, and 70%, then fixed in 3.7% Paraformaldehyde. After washing, slides were incubated in mixture of TdT, Mn+2, and TdT dNTP for 1 hour at 37°C. The reaction was stopped with TdT Stop Buffer for 5 minutes. After washing with deionized water, the slides were incubated with Streptavidin-HRP (diluted 1:200) solution for 20 minutes at room temperature. Slides were counter-stained, mounted, covered with coverslips and visualized by confocal microscopy (LSM 510, Carl Zeiss, Inc). Apoptotic cells were identified as doubly labeled with TdT Fluorescein and propidium iodide and only nuclei that were clearly labeled yellow were scored.

Measurement of oxidative and nitrosative stress

The production of superoxide as oxidative stress, and peroxynitrite as nitrosative stress were measured in frozen eye sections using the oxidative fluorescent dye dihydroethidium (DHE) and nitrotyrosine immunofluorescent staining, respectively. DHE (2 μM) (Sigma- Aldrich, Oakville, ON, Canada) was applied to 7μm thick eye sections and the slides were then incubated in a light protected humidified chamber at 37 ºC for 30 min. Cells are permeable to DHE. In the presence of superoxide, DHE is oxidized to fluorescent ethidium, which is trapped by intercalation with DNA. Ethidium is excited at 518 nm with an emission spectrum of 605 nm. The intensity of the fluorescence was quantified by Image J software (version 1.42; National Institutes of Health, Bethesda, MD). Nitrotyrosine levels in frozen eye sections were quantified by immunofluorescent histochemistry. Sections were stained with antibody for nitrotyrosine (Santa Cruz Biotechnology, Santa Cruz, CA). Images were observed using fluorescent microscope.

Data Analysis

The results are expressed as mean ± SD. Differences among experimental groups were evaluated by analysis of variance, and the significance of differences between groups was assessed by the posthoc test (Fisher's PLSD). Significance was defined as $P < 0.05$.

Results

Inhibition of adenosine kinase mitigates retinal inflammation in mice with TON

Inflammation has been proposed to be important in the pathogenesis of TON. An early feature of inflammation is the activation of microglia, release of cytokines leading to increased ganglion cell death. Consistently, TNF-α and IL-6 expressions were markedly increased in the retinas of 8-week diabetic mice as compared with normal, non-diabetics as revealed by qRT-PCR (**Figure 1**). Treatment with ABT 702 (1.5mg/kg i.p., twice a week) reduced these expressions.

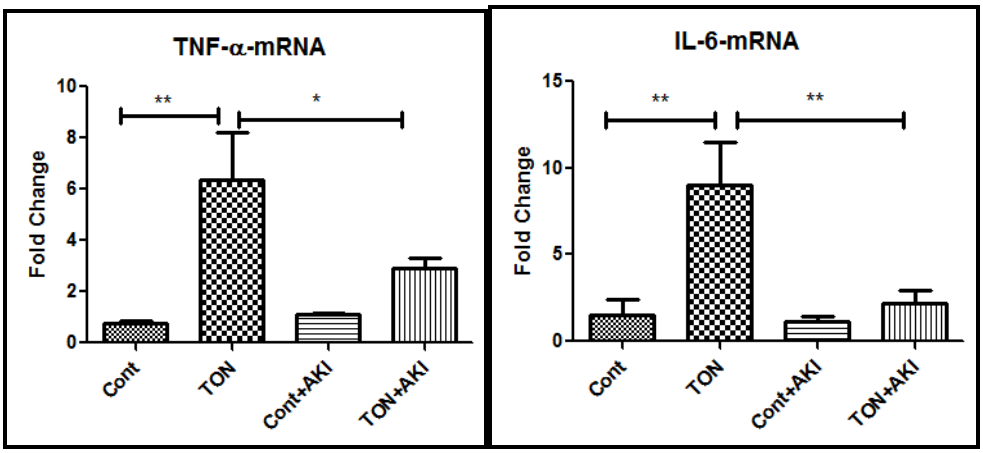


Figure 1. ABT702 treatment of mouse model of TON reduces mRNA expression of proinflammatory cytokines.

Inhibition of adenosine kinase blocks $A_{2A}AR$ up-regulation in mice with TON

$A_{2A}AR$ is the most likely candidate for mediating the anti-inflammatory effect of adenosine (Milne and Palmer, 2011). Inflammation is associated with up-regulation of $A_{2A}AR$ (Pang et al., 2010). The increased $A_{2A}AR$ expression may possibly represent an endogenous mechanism to combat the inflammation associated with injury. Consistent with this, TON induced up-regulation of $A_{2A}AR$ in the retina as compared with normal (**Figure 2**). Treatment of ABT 702 reduced $A_{2A}AR$ expression in the mice with TON as compared with vehicle-treated mice with TON (**Figure 2**).

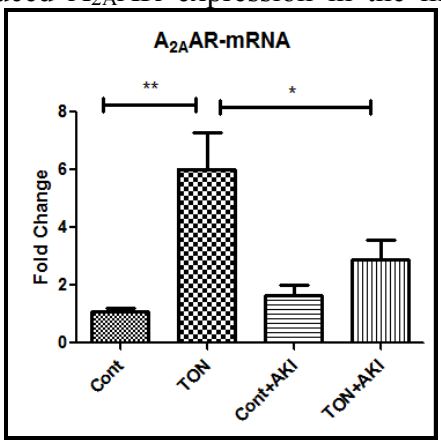


Figure 2. ABT702 treatment of mouse model of TON reduces up-regulation of $A_{2A}AR$.

Inhibition of adenosine kinase blocks ENT1 up-regulation in mice with TON

ENT1 plays an integral role in adenosine function in inflammation by regulating adenosine levels in the vicinity of adenosine receptors. Hyperglycemia up-regulated ENT1 expression and adenosine transport in cultured human aortic smooth muscle cells (Leung et al., 2005). Consistent with this observation, TON induced up-regulation of ENT1 in the retina as compared with normal (**Figure 3**). The increase in ENT1 activity in TON may affect the availability of adenosine in the vicinity of adenosine receptors. Treatment with ABT 702 reduced ENT1 expression in these mice as compared with vehicle-treated mice with TON (**Figure 3**).

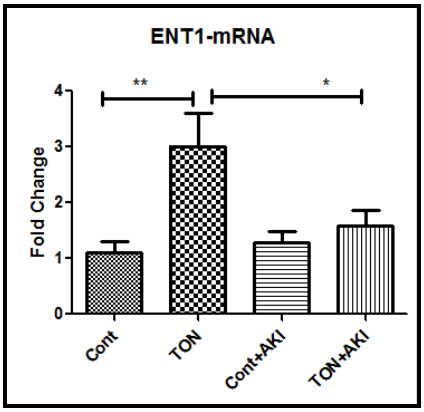
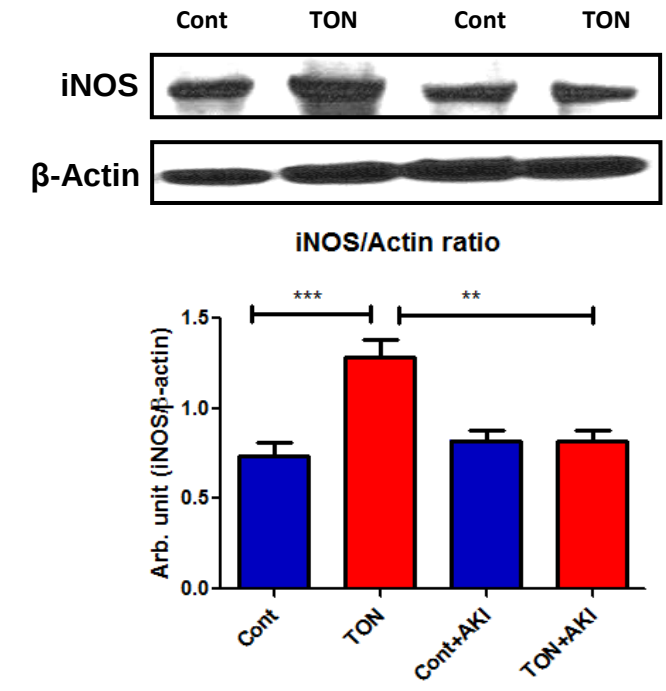
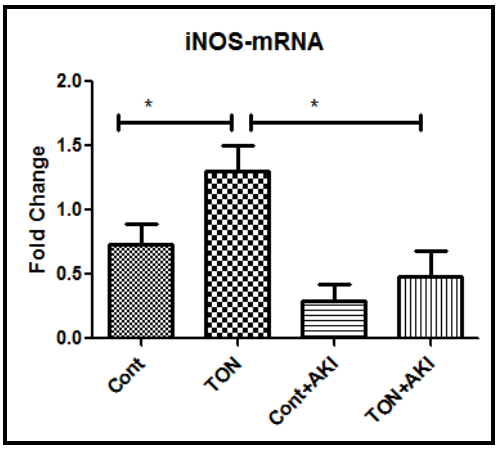


Figure 3. ABT702 treatment of mouse model of TON reduces up-regulation of ENT1.

Inhibition of adenosine kinase mitigates oxidative and nitrosative stress in the retina of TON mice

Mice with TON showed a significant increase in DHE staining as compared with normal group and treatment with ABT 702 reduced DHE staining in TON mice retinas as compared with vehicle-treated TON mice (**Figure 4**). In addition, immunofluorescent staining of nitrotyrosine, a stable product formed from the reaction of peroxynitrite with tyrosine residues and an index of nitrosative damage, was elevated in the retinas of TON mice as compared with that in normal retinas. ABT702-treated TON mice showed decreased nitrotyrosine staining as compared with vehicle-treated TON mice (**Figure 4**).





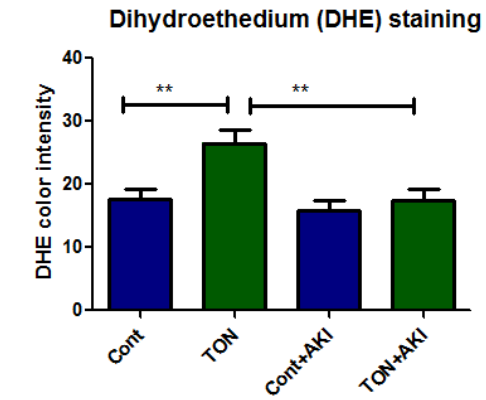
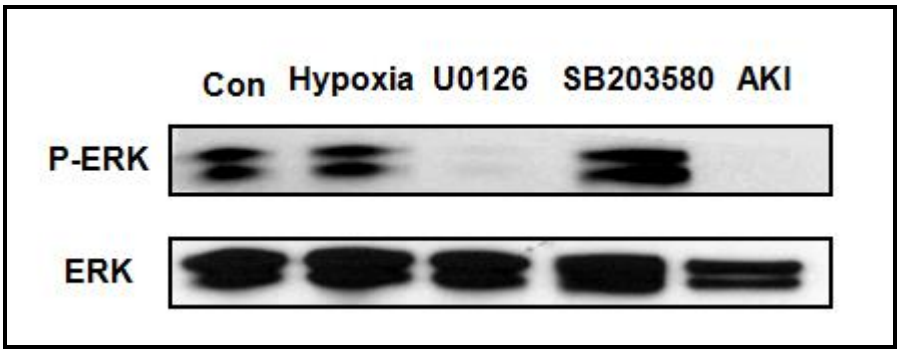


Figure 4. ABT702 treatment of mouse model of TON reduces up-regulation of oxidative and nitrosative stresses.

Inhibition of adenosine kinase mitigates MAPKinases in hypoxia-treated retinal microglial cells
ABT 702 attenuated p-ERK1/2 activation, but not p-P38, in hypoxia-treated retinal microglial cells (**Figure 5**). These results suggest that optic nerve injury-induced retinal inflammation is mediated by ERK.



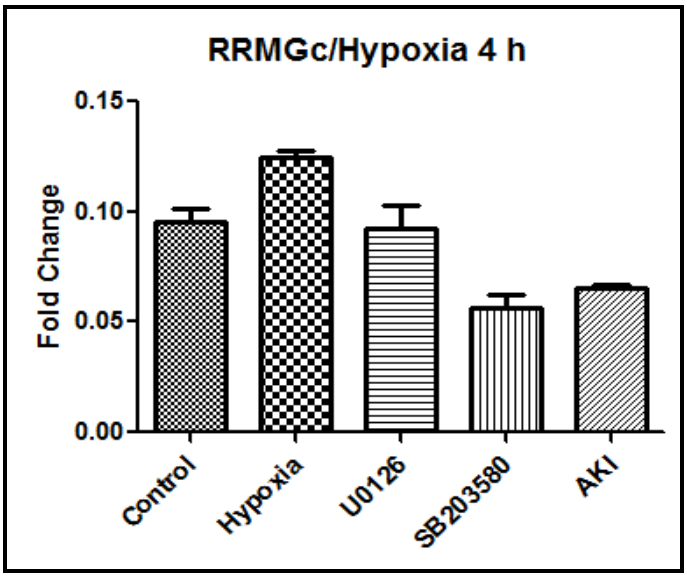


Figure 5. ABT 702 attenuated p-ERK1/2 activation, but not p-P38, in hypoxia-treated retinal microglial cells.

KEY RESEARCH ACCOMPLISHMENTS

The results of the present investigation suggested that ABT-702 had a protective role against marked TON-induced retinal inflammation and damage by augmenting the endogenous therapeutic effects of site- and event-specific accumulation of extracellular adenosine.

REPORTABLE OUTCOMES

This research has resulted in publications in J Neuroimmunol [10] and Life Science [11].

CONCLUSION

Based on our findings, we conclude that Adenosine Kinase inhibition may have potential role in regulating inflammatory mechanism in TON.

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APPENDICES

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ABT-702, an adenosine kinase inhibitor, attenuates inflammation in diabetic retinopathy

Nehal M. Elsherbiny [a,c], Saif Ahmad [a], Mohammad Naime [a], Ahmed M. Elsherbini [a], Sadanand Fulzele [b], Mohammed M. Al-Gayyar [c], Laila A. Eissa [c], Mamdouh M. El-Shishtawy [c], Gregory I. Liou [a,*]

[a] Department of Ophthalmology, Georgia Regents University, 30909, USA
[b] Department of Orthopedics, Georgia Regents University, 30909, USA
[c] Department of Clinical Biochemistry, Faculty of Pharmacy, Mansoura University, 35516, Egypt

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ABSTRACT

Aims: This study was undertaken to determine the effect of an adenosine kinase inhibitor (AKI) in diabetic retinopathy (DR). We have shown previously that adenosine signaling via A_{2A} receptors (A_{2A}AR) is involved in retinal protection from diabetes-induced inflammation. Here we demonstrate that AKI-enhanced adenosine signaling provides protection from DR in mice.

Main methods: We targeted AK, the key enzyme in adenosine metabolism, using a treatment regime with the selective AKI, ABT-702 (1.5 mg/kg intraperitoneally twice a week) commencing at the beginning of streptozotocin-induced diabetes at the age of eight weeks. This treatment, previously demonstrated to increase free adenosine levels in vivo, was maintained until the age of 16 weeks. Retinal inflammation was evaluated using Western blot, Real-Time PCR and immuno-staining analyses. Role of A_{2A}AR signaling in the anti-inflammation effect of ABT-702 was analyzed in Amadori-glycated-albumin (AGA)-treated microglial cells.

Key findings: At 16 weeks, when diabetic mice exhibit significant signs of retinal inflammation including up-regulation of oxidative/nitrosative stress, A_{2A}AR, ENT1, Iba1, TNF-α, ICAM1, retinal cell death, and down-regulation of AK, the ABT-702 treated group showed lower signs of inflammation compared to control animals receiving the vehicle. The involvement of adenosine signaling in the anti-inflammation effect of ABT-702 was supported by the TNF-α release blocking effect of A_{2A}AR antagonist in AGA-treated microglial cells.

Significance: These results suggest a role for AK in regulating adenosine receptor signaling in the retina. Inhibition of AK potentially amplifies the therapeutic effects of site- and event-specific accumulation of extracellular adenosine, which is of highly translational impact.

Introduction

Diabetic retinopathy (DR) is the leading cause of acquired vision loss among adults of working age in developed countries worldwide and has been perceived as the most common microvascular complication of diabetes (Zhu and Zou, 2012). Despite many years of research, treatment options for DR, including photocoagulation, vitrectomy and repeated intraocular injections of steroids and anti-vascular endothelial growth factor (VEGF), remain invasive, limited and with adverse effects. This is because VEGF, although induces angiogenesis, is also required for the maintenance of retinal neurons. By neutralizing VEGF with anti-VEGF, angiogenesis could be solved at the expense of neuronal degeneration. Therefore, there is a great need for the development of new non-invasive therapies.

The early signs of DR in experimental diabetic models include vascular inflammatory reactions due to oxidative stress, pro-inflammatory cytokines, and the consequent upregulation of leukocyte adhesion molecules (Tang and Kern, 2011). These reactions lead to breakdown of the blood–retinal barrier, vascular occlusion and tissue ischemia, which in turn leads to neuronal cell death (El-Remessy et al., 2006). Under these conditions, normally quiescent microglial cells become activated. Activated microglia release reactive oxygen species and proinflammatory mediators, such as tumor necrosis factor TNF-α (Xie et al., 2002). Thus, research on retinal microglia activation may provide insights into the pathogenesis of DR (Ibrahim et al., 2011a).

Adenosine is centrally involved in the signaling cascade of related events, including anti-inflammatory actions, angiogenesis, oxygen supply/demand ratio, and ischemic pre- and postconditioning (Johnston-Cox and Ravid, 2011). Under these circumstances, the local levels of extracellular adenosine are increased due to the increased need for energy supplied by ATP (Vallon et al., 2006). The increased extracellular adenosine at inflamed sites can protect against cellular damage by activating the A_{2A} adenosine receptor (A_{2A}AR), a Gs-coupled

* Corresponding author at: Department of Ophthalmology, Georgia Health Sciences University, 1120 15th Street, Augusta, GA 30912, USA. Tel.: +1 706 721 4599.
E-mail address: giliou@gru.edu (G.I. Liou).

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receptor (Ibrahim et al., 2011b). Extracellular adenosine re-uptake by the equilibrative and concentrative nucleoside transporters (ENT and CNT) allows for adenosine conversion to AMP by adenosine kinase (AK) (Löffler et al., 2007), decreases extracellular adenosine levels, and terminates the protective effect of A_{2A}AR. The removal of extracellular adenosine is predominantly regulated by AK via conversion of adenosine into AMP. The extracellular levels of adenosine are largely dependent on the intracellular activity of AK whereas the degradation of adenosine into inosine by adenosine deaminase (ADA) plays only a minor role in regulating adenosinergic function (Pak et al., 1994).

We aim to evaluate the AK in regulating adenosine signaling in the retina. It was reported that the degree of brain injury directly depends on expression levels of AK and the resulting extracellular levels of adenosine (Boison, 2006). Indeed, transgenic mice overexpressing AK are highly susceptible to stroke-induced brain injury (Shen et al., 2011). We therefore hypothesized that adenosine kinase inhibitors (AKI) could play the same protective role in the diabetic retina.

Methods

Preparation of AKI

A selective AKI, 4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin-3-yl)pyrido[2,3-dipyrimidine (ABT-702, 5 mg) from Santa Cruz was dissolved in 0.25 mL of DMSO (20 mg/mL) and then in 9.75 mL of distilled water to prepare a 0.5 mg/mL stock solution. The solutions were aliquoted and stored at −20 °C for later use. An equivalent volume of vehicle solution was administered to the control animals. ABT-702 was used previously to study the effect of AK inhibition on neuronal inflammation (Suzuki et al., 2001) and age-related hearing loss (Vlajkovic et al., 2011). ABT-702 was 1300- to 7700-fold selective for AK compared with a number of other neurotransmitter and peptide receptors, ion channel proteins, neurotransmitter/nucleoside reuptake sites, and enzymes, including cycloxygenases-1 and -2 (Jarvis et al., 2000).

Animal preparation and experimental design

All procedures with animals were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare, National Institutes of Health Publication No. 80-23) and the Georgia Health Sciences University guidelines. Male, eight-week-old mice in C57BL/6J (Jackson Laboratory, Bar Harbor, ME) background were used. Animals were given i.p. injections of vehicle or freshly prepared streptozotocin in 0.01 mol/L sodium citrate buffer, pH 4.5 (45 mg/kg) after a 4-hour fast each day for 5 consecutive days. Diabetes was confirmed by fasting blood glucose levels >250 mg/dL. The diabetic and normal, non-diabetic mice were randomly divided into four subgroups: ABT-702-treated diabetic, ABT-702-treated normal, vehicle-treated diabetic and vehicle-treated normal (1.5 mg/kg intraperitoneally, twice a week).

Eight weeks after the establishment of diabetes, the retinas were removed, snap frozen in liquid nitrogen, stored at −80 °C, and analyzed by Quantitative Real Time-PCR (qRT–PCR) or Western blot. Frozen eye sections were prepared for immunofluorescence or immunohistochemistry.

Measurement of blood glucose

Blood glucose was measured by blood glucose meter (OneTouch UltraEasy, USA).

Primary retinal microglia culture

Microglial cells were isolated from retinas of newborn Sprague Dawley (SD) rats according to a previous procedure (El-Remessy et al., 2008) with minor modifications. Briefly, retinas were collected into phosphate-buffered saline and digested with 0.125% trypsin for 3–5 min before mixing with Dulbecco's Modified Eagle Medium (DMEM)/F12 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Retina pieces were then filtered through a mesh (100 μm), collected by centrifugation, resuspended in culture medium and plated onto T75 cell culture flasks (Corning, NY) at a density of 2×10^5 cells/cm^2. After 2 weeks, microglial cells were harvested by shaking the flasks at 100 rpm for 1 h. Immunocytochemical studies showed that more than 95% cultured cells stained positively for Iba1. Almost none of these cells showed positive staining for GFAP, indicating that majority of the isolated cells were microglia and were not contaminated with astrocytes or Müller cells (data not shown).

Drug treatment effects on cultured microglial cells

Microglial cells were seeded at a density of 5×10^5 cells/well in a collegen-1-pretreated 24-well tissue culture plate. One day after seeding, the cultured wells were washed with Cellgro Complete (Mediatech, Manassas, VA) and incubated in the same media with various treatments. Cells were pretreated with AR antagonists (all are from Sigma-Aldrich except ZM 241385, which is from Tocris) at the indicated concentrations for 30 min at 37 °C, followed with ABT-702 or vehicle for 30 min at 37 °C. Microglial activation was then achieved by addition of Amadori-glycated albumin (AGA; Sigma) with undetectable endotoxin (<0.125 units/mL, 10 EU = 1 ng lipopolysaccharide; Lonza, Basel, Switzerland) (Ibrahim et al., 2011a) to each well at a final concentration of 250 or 500 μg/mL at indicated time points (Ibrahim et al., 2011a,b). After the indicated time course, culture media were collected and assayed for TNF-α by ELISA.

ELISA for TNF-α

TNF-α levels in the supernatants of culture media were estimated with ELISA kits (R & D, Minneapolis, MN) per the manufacturer's instructions. Standards and samples were added and bound by the immobilized antibody. After washing an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells followed by a substrate solution yielding a colored product. The intensity of the color was measured at 450 nm. The sample levels were calculated from the standard curve and corrected for protein concentration.

Quantitative real time-PCR

Total RNA was isolated from mouse retina using SV Total RNA Isolation kit (Promega, Madison, WI) following manufacturer's instructions, and the quality of the RNA preparations was monitored by absorbance at 260 and 280 nm (Helios-Gamma, Thermo Spectronic, Rochester, NY). The RNA was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using iScript reagents from Bio-Rad on a programmable thermal cycler (PCR-Sprint, Thermo Electron, Milford, MA). Fifty ng of cDNA was amplified in each qRT-PCR using a Bio-Rad iCycler, ABgene reagents (Fisher scientific) and appropriate primers (Table 2). Average of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA was used as the internal control for normalization.

Western blot analysis

Dissected individual mouse retinas were homogenized in modified RIPA buffer (Upstate, Lake Placid, NY), containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% deoxycholate, supplemented with 40 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride and 1:100 (v/v) of proteinase inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation at 12,000 ×g at 4 °C for 30 min. Protein was determined by DC Protein

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Table 1
Body weight and blood glucose levels in studied groups.

Group	No	Body weight (g)		Blood glucose (mg/dL)	
Non-diabetic	7	30.8	0.59	198.6	11.61
Non-diabetic + ABT 7002	7	30.0	0.67	198.6	7.93
Diabetic	7	24.1**	0.61	399.6***	11.22
Diabetic + ABT 702	7	23.4**	0.57	373.5***	6.82

Mean ± SD.
** $P < 0.001$ vs non-diabetic group.
*** $P < 0.0001$ vs non-diabetic group.

Assay (Bio-Rad, Hercules, CA) and 100 μg was boiled in Laemmli sample buffer, separated by SDS-PAGE on a gradient gel (4 to 20%) (Pierce, Rockford, IL), transferred to nitrocellulose membrane and incubated with specific antibodies. Antibodies for β-actin, ICAM-1, ENT1, A_{2A}AR and AK (Santa Cruz Biotechnology, Santa Cruz, CA) were detected with a horseradish peroxidase-conjugated antibody and enhanced chemiluminescence (ECL) (Amersham BioSciences, Buckinghamshire, UK). The same filter was re-probed with control antibodies, such as those for the actin. Intensity of immunoreactivity was measured by densitometry.

Immunolocalization studies

Immunofluorescence analysis was performed using frozen eye sections. Briefly, cryostat sections (7 μm) were fixed in 4% paraformaldehyde, blocked with Dako protein block serum-free and then incubated overnight at 4 °C with primary antibodies: rabbit anti-Iba-1 (Proteintech Group), or goat anti-ICAM-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-AK (Santa Cruz Biotechnology, Santa Cruz, CA). Thereafter, sections were briefly washed with PBS and incubated with appropriate secondary antibodies. Slides were examined by fluorescent microscope. Specificity of the reaction was confirmed by omitting the primary antibody, or by using non-immune IgG.

Immunohistochemistry of cleaved, activated caspase-3 was performed as follows. Retinas frozen sections were fixed in 4% paraformaldehyde, rinsed in PBS, blocked with 0.3% H_2O_2 then Mouse on Mouse (M.O.M.) Immunoglobulin Blocking Solution (Vector Laboratories, Burlingame, CA), and reacted with antibodies detecting cleaved, activated caspase-3 (Cell Signaling Technology) for 16 to 20 h at room temperature. Sections were washed, and reacted with M.O.M. biotinylated anti-mouse Ig reagent (1:250), followed by M.O.M. ABC reagent. Color was developed with 3,3′-diaminobenzidine (DAB) as substrate.

Terminal dUTP nick end-labeling (TUNEL)

TUNEL was performed in frozen sections using the TACS-2 TdT Fluorescein In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD) counter-stained with propidium iodide, according to the manufacturer's suggestions. Briefly, sections were hydrated with alcohol 100%, 95%, and 70%, and then fixed in 3.7% paraformaldehyde. After washing, slides were incubated in mixture of TdT, Mn + 2, and TdT dNTP for 1 h at 37 °C. The reaction was stopped with TdT Stop Buffer for 5 min. After washing with deionized water, the slides were incubated with Streptavidin–HRP (diluted 1:200) solution for 20 min at room temperature. Slides were counter-stained, mounted, covered with coverslips and visualized by confocal microscopy (LSM 510, Carl Zeiss, Inc.). Apoptotic cells were identified as doubly labeled with TdT Fluorescein and propidium iodide and only nuclei that were clearly labeled yellow were scored.

Table 2
The primer sets used for the detection of mouse genes by quantitative Real-Time PCR analysis.

Gene	Primer sequence (5′–3′)	Accession number
TNF-α	CCCTCACACTCAGATCATCTTCT GTCACGACGTGGGCTACAG	NM_013693.2
ICAM-1	CGCTGTGCTTTGAGAACTGTG ATACACGGTGATGGTAGCGGA	NM_010493
Iba-1	GTCCTTGAAGCGAATGCTGG CATTCTCAAGATGGCAGATC	NM_019467
GAPDH	CAT GGC CTC CAA GGA GTAAGA GAG GGA GAT GCT CAG TGT TGG	M32599
18S	AGT GCG GGT CAT AAG CTT GC GGG CCT CAC TAA ACC ATC CA	NR_003278

Measurement of oxidative and nitrosative stress

The production of superoxide as oxidative stress, and peroxynitrite as nitrosative stress were measured in frozen eye sections using the oxidative fluorescent dye dihydroethidium (DHE) and nitrotyrosine immunofluorescent staining, respectively. DHE (2 μM) (Sigma-Aldrich, Oakville, ON, Canada) was applied to 7 μm thick eye sections and the slides were then incubated in a light protected humidified chamber at 37 °C for 30 min. Cells are permeable to DHE. In the presence of superoxide, DHE is oxidized to fluorescent ethidium, which is trapped by intercalation with DNA. Ethidium is excited at 518 nm with an emission spectrum of 605 nm. The intensity of the fluorescence was quantified by Image J software (version 1.42; National Institutes of Health, Bethesda, MD). Nitrotyrosine levels in frozen eye sections were quantified by immunofluorescent histochemistry. Sections were stained with antibody for nitrotyrosine (Santa Cruz Biotechnology, Santa Cruz, CA). Images were observed using fluorescent microscope.

Data analysis

The results are expressed as mean ± SD. Differences among experimental groups were evaluated by analysis of variance, and the significance of differences between groups was assessed by the posthoc test (Fisher's PLSD). Significance was defined as $P < 0.05$.

Results

Body weight and blood glucose levels in studied group

The final body weight was lower after streptozotocin injection, and it was not affected by ABT 702 treatment. Blood glucose levels were higher in diabetic mice compared with non-diabetic groups and they were not modified by ABT-702 treatment (Table 1).

Inhibition of adenosine kinase mitigates retinal inflammation in diabetic mice

Inflammation has been proposed to be important in the pathogenesis of DR. An early feature of inflammation is the release of cytokines leading to increased expression of endothelial activation markers such as Intercellular Adhesion Molecule 1 (ICAM-1) (Rangasamy et al., 2012). Consistently, ICAM-1 and TNF-α expressions were markedly increased in the retinas of 8-week diabetic mice as compared with normal, non-diabetics as revealed by qRT-PCR (Fig. 1A, B) and Western analyses (Fig. 1C). Treatment with ABT 702 (1.5 mg/kg i.p., twice a week) reduced retinal ICAM-1 expression and retinal TNF-α in the diabetic mice as compared with vehicle-treated diabetic mice.

Inhibition of adenosine kinase blocks A_{2A}AR up-regulation in diabetic mice

A_{2A}AR is the most likely candidate for mediating the anti-inflammatory effect of adenosine (Milne and Palmer, 2011). Diabetes

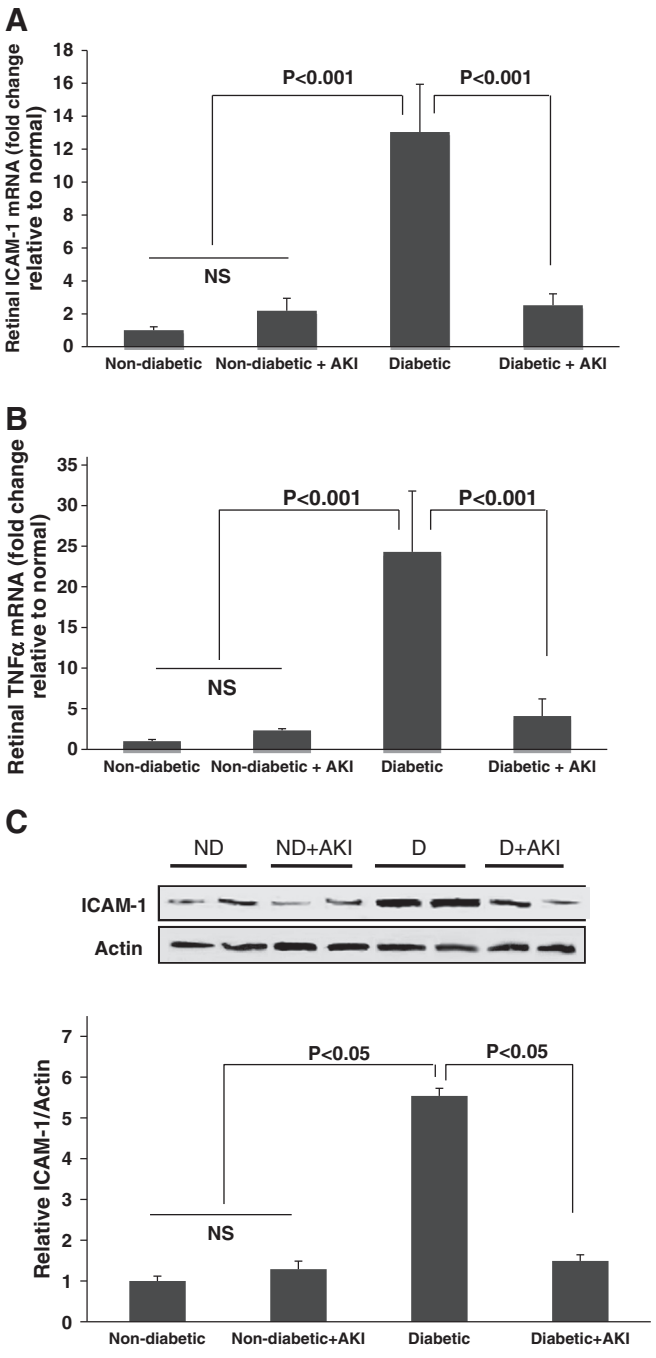

Fig. 1. Inhibition of adenosine kinase mitigates retinal inflammation in diabetic mice. A, B) Retinal expression of ICAM1 and TNF-α measured by R-T PCR. A) Effect of AK inhibition on ICAM1 expression in the diabetic mouse retina. B) Effect of AK inhibition on TNF-α expression in the diabetic mouse retina. GAPDH and 18S were used as reporter genes. The results represent the means ± SD of fold changes calculated using expression level, normalized to the level of the normal non-diabetic mice (n = 4–6). C) Representative Western blots and quantitative analysis of retinal ICAM1 expression showing the effect of AK inhibition on ICAM-1 expression in the diabetic mouse retina (n = 4).

or inflammation is associated with up-regulation of A_{2A}AR (Pang et al., 2010). The increased A_{2A}AR expression may possibly represent an endogenous mechanism to combat the inflammation associated with diabetes induction. Consistent with this, diabetes induced up-regulation of A_{2A}AR in the retina as compared with normal (Fig. 2). Treatment of ABT 702 reduced A_{2A}AR expression in the diabetic mice as compared with vehicle-treated diabetic mice (Fig. 2).

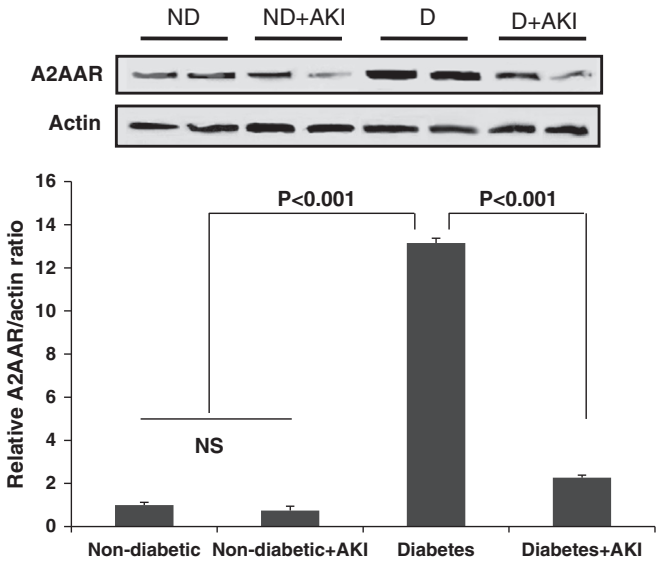

Fig. 2. Inhibition of adenosine kinase blocks A2AAR up-regulation in diabetic mice. Representative Western blots and quantitative analysis of retinal A_{2A}AR expression showing the effect of AK inhibition on A_{2A}AR expression in the diabetic mouse retina (n = 4).

Inhibition of adenosine kinase blocks ENT1 up-regulation in diabetic mice

ENT1 plays an integral role in adenosine function in diabetes by regulating adenosine levels in the vicinity of adenosine receptors. Hyperglycemia up-regulated ENT1 expression and adenosine transport in cultured human aortic smooth muscle cells (Leung et al., 2005). Consistent with this observation, diabetes induced up-regulation of ENT1 in the retina as compared with normal (Fig. 3). The increase in ENT1 activity in diabetes may affect the availability of adenosine in the vicinity of adenosine receptors and, thus, alter vascular functions in diabetes. Treatment with ABT 702 reduced ENT1 expression in diabetic mice as compared with vehicle-treated diabetic mice (Fig. 3).

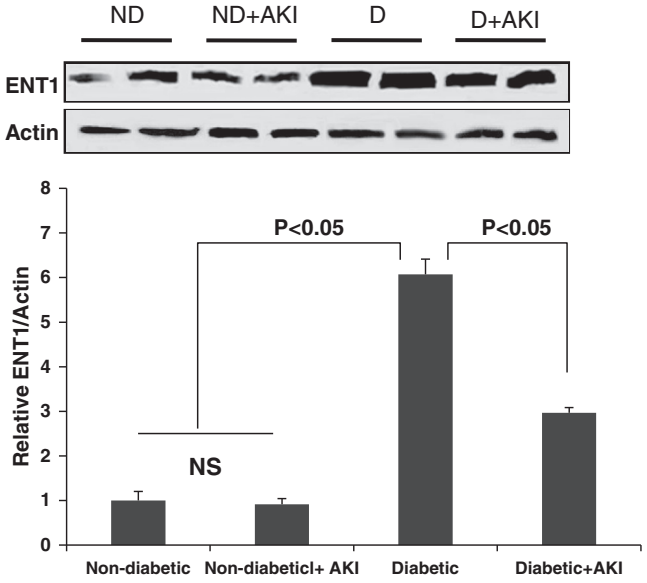

Fig. 3. Inhibition of adenosine kinase blocks ENT1 up-regulation in diabetic mice. Representative Western blots and quantitative Western analysis of retinal ENT1 expression showing the effect of AK inhibition on ENT1 expression in the diabetic mouse retina (n = 4).

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Inhibition of adenosine kinase blocks adenosine kinase down-regulation in diabetic mice

Sakowicz and Pawelczyk reported reduced AK activity in tissues of diabetic rat. They suggested that the expression of AK to some extent is controlled by insulin. Reduced AK expression is also reported in hypoxic tissues (Morote-Garcia et al., 2008). The reduced AK expression may possibly represent an endogenous protective mechanism to raise extracellular adenosine levels. Consistent with these observations, AK expression was reduced in retinas of diabetic mice as compared with the normal (Fig. 4A, B). Treatment with ABT 702 blocked the diabetic effect on AK in diabetic mice as compared with vehicle-treated diabetic mice (Fig. 4A, B).

Inhibition of adenosine kinase mitigates retinal microglial activation in diabetic mice

We next sought to explore a potential mechanism by which ABT702 regulates inflammation in DR. Through immunofluorescence, the effect of ABT702 treatment on microglial activation was determined by measuring Iba1 expression, which is up-regulated in activated microglia in diabetic mice as compared with normal. Iba1 was found to be decreased in the AKI-treated diabetic mice as compared with vehicle-treated diabetic mice (Fig. 5A). In addition, the level of Iba1 mRNA was markedly reduced in the retinas of AKI-treated diabetic mice as compared with vehicle-treated diabetic mice (Fig. 5B).

Inhibition of adenosine kinase mitigates oxidative and nitrosative stress in the retina of diabetic mice

Oxidative stress is a key pathogenic factor in DR (Madsen-Bouterse and Kowluru, 2008). Diabetic mice showed a significant increase in DHE staining as compared with normal group and treatment with ABT 702 reduced DHE staining in diabetic mice retinas as compared with vehicle-treated diabetic mice (Fig. 6A). In addition, immunofluorescent staining of nitrotyrosine, a stable product formed from the reaction of peroxynitrite with tyrosine residues and an index of nitrosative damage, was elevated in the retinas of diabetic mice as compared with that in normal retinas. ABT702-treated diabetic mice showed decreased nitrotyrosine staining as compared with vehicle-treated diabetic mice (Fig. 6B).

Inhibition of adenosine kinase reduces retinal cell death in diabetic mice

Retinal cell death in diabetic and non-diabetic animals treated and untreated with ABT702 was determined by immunostaining of cleaved,

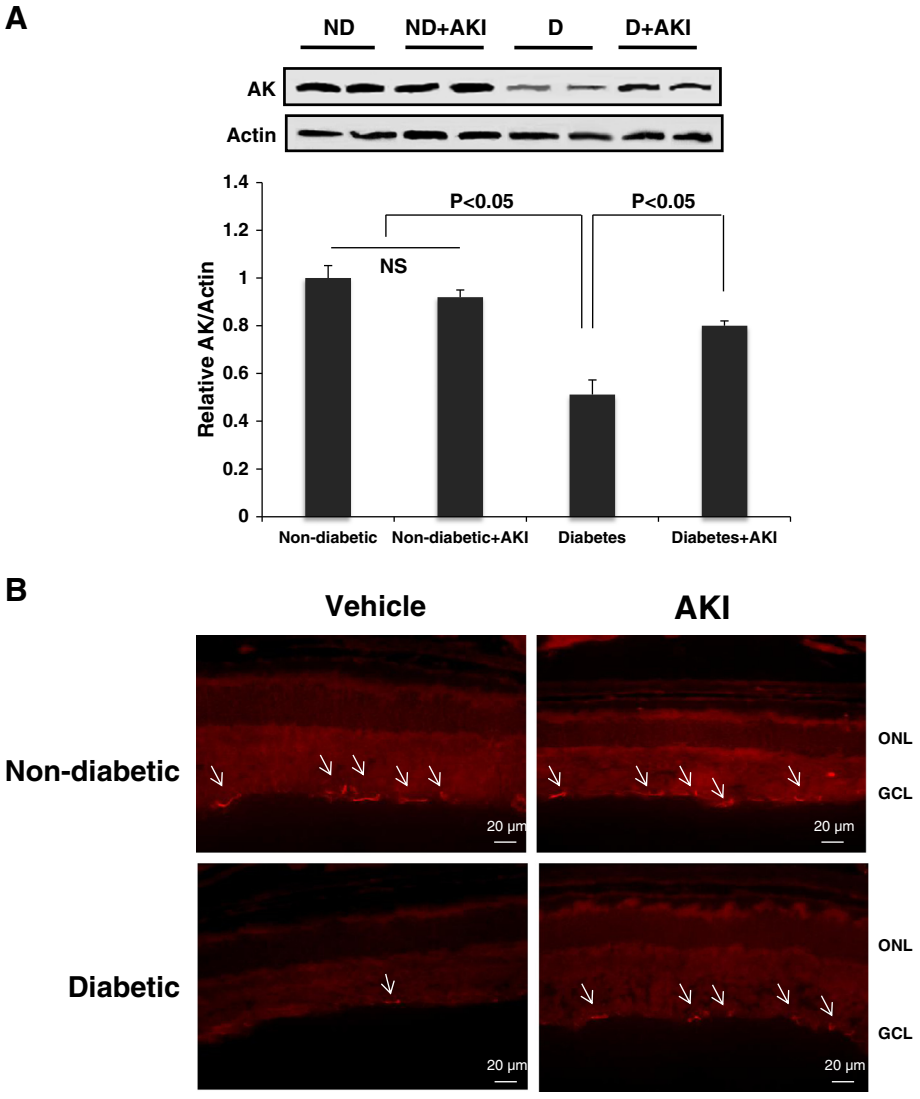

Fig. 4. Inhibition of adenosine kinase blocks adenosine kinase down-regulation in diabetic mice. A) Western blot analysis. B) Immunofluorescence staining. Arrows indicate AK distribution (n = 4–6).

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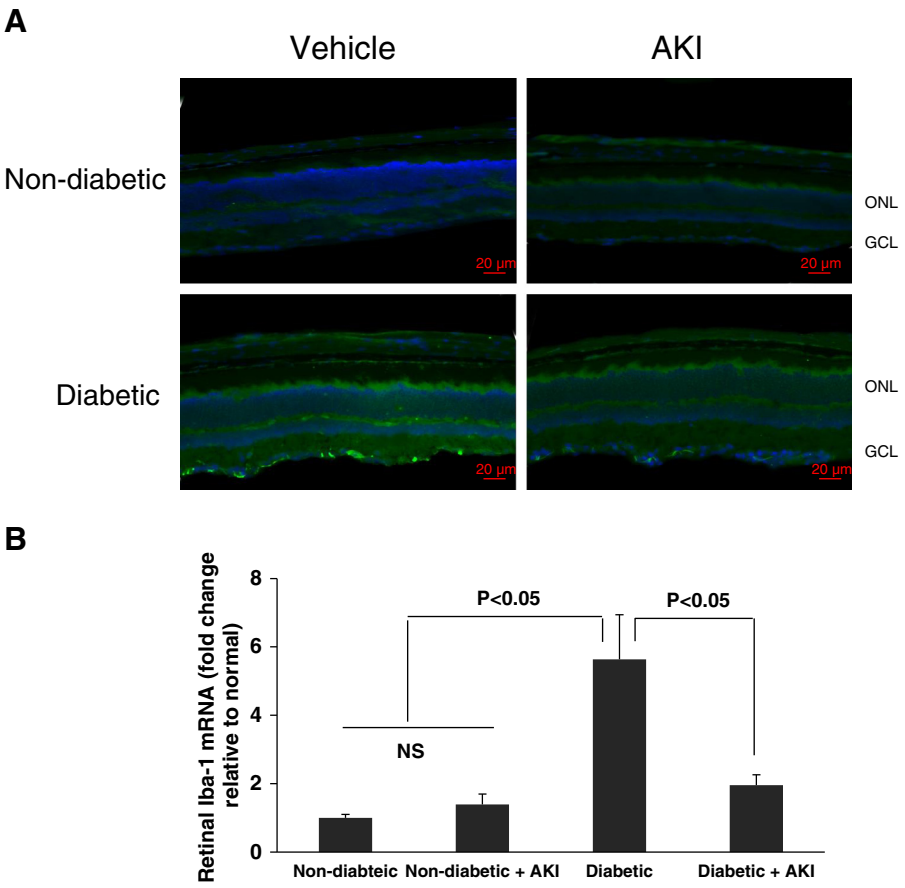

Fig. 5. Inhibition of adenosine kinase mitigates retinal microglial activation in diabetic mice. A) Effect of AK inhibition on Iba1 expression in the diabetic mouse retina determined by immunofluorescence staining. Scale bar: 20 μm; B) determined by RT PCR analysis; GAPDH and 18S were used as reporter genes. The results represent the means ± SE of fold changes calculated using expression level, normalized to the level of the normal non-diabetic mice (n = 4–6).

activated caspase-3, a known marker for apoptosis, and by TUNEL. As shown by these methods, increased cell death appeared in the retinal ganglion cell layer of diabetic animals (Fig. 7A, B). Treatment with ABT702 blocked cell death in diabetic mice but did not affect treated normal controls (Fig. 7A, B). Taken together, the above findings suggest that AK inhibition plays a role in attenuating retinal oxidative stress, inflammation, and cell death by dampening microglial cell activation.

Inhibition of adenosine kinase is more effective than adenosine deaminase in blocking TNF-α release in activated retinal microglial cells

The extracellular levels of adenosine are largely dependent on the intracellular activity of AK whereas the degradation of adenosine into inosine by adenosine deaminase (ADA) plays only a minor role in regulating adenosinergic function (Pak et al., 1994). This was further confirmed by others: endogenous adenosine levels in the brain are mainly dependent on the activity of AK (Gouder et al., 2004). To compare the anti-inflammatory effect of the inhibitors of AK and ADA, we developed a cultured retinal microglia model. This model also helps elucidate the molecular mechanisms responsible for this effect. In this model, we determined the ability of ABT702 and EHNA, an ADA inhibitor, to affect TNF-α release in retinal microglia in response to AGA treatment. EHNA at levels comparable to the present study was previously used to study the cardioprotective effect of adenosine metabolism inhibitors (Peart et al., 2001). Microglial cells were pretreated with the indicated concentrations of ABT702 and EHNA for 1/2 h then treated with AGA for 16 h. The supernatants were collected and assayed for TNF-α by ELISA. As shown (Fig. 8), ABT 702 inhibited AGA-induced TNF-α release in a dose-dependent manner more significantly than EHNA.

Inhibition of adenosine kinase blocks TNF-α release via A_{2A}AR

To identify the AR subtype(s) involved in ABT 702 inhibitory effect on TNF-α release in the retinal microglia in response to AGA, we examined the effect of the ABT 702 in the presence of AR subtype-selective antagonists. The concentrations of each antagonist chosen for this study were based on the affinity and selectivity for the recombinant mouse AR subtypes determined by radioligand binding studies, and was applied to rat retinal microglial cells previously (Liou et al., 2008; Ibrahim et al., 2011b). As shown in Fig. 9, cells pretreated with vehicle showed a significant increase in AGA-induced TNF-α release compared with vehicle-treated control cells. Treatment with ABT 702 at a concentration of 20 μM potently inhibited AGA-induced TNF-α release. When the cells were pretreated with the A_{1A}R antagonist 1,3-dipropyl-8-cyclopentylxanthine (CPX; 100 nM), the A_{2B}AR antagonist 8-[4-[((4-cyanophenyl) arbamoylmethyl) oxy] phenyl]-1,3-di(n-propyl) xanthine hydrate (MRS 1754; 1 μM), or the A_{3A}R antagonist 3-propyl-6-ethyl-5[(ethylthio)carbonyl]-2-phenyl-4- propyl-3-pyridine-carboxylate (MRS 1523; 10 μM), the inhibitory effect of ABT 702 on TNF-α release was not affected. However, this effect was successfully blocked by 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-α][1,3,5]triazin-5-ylamino]ethyl} phenol (ZM 241385) at concentrations (100 and 500 nM) capable of blocking A_{2A}ARs. These results suggest that ABT 702 inhibited AGA-induced TNF-α release from retinal microglia via the A_{2A}AR.

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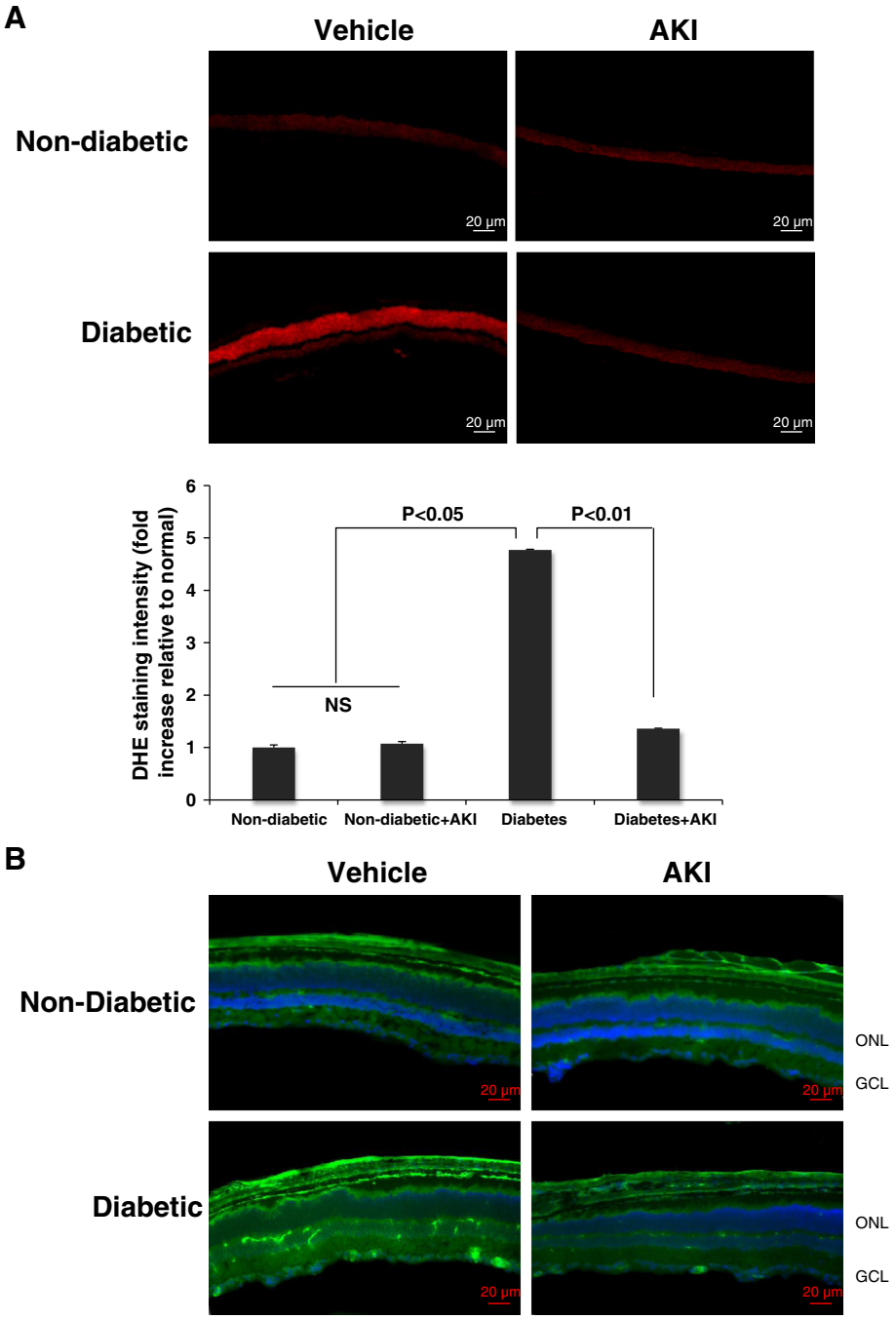

Fig. 6. Inhibition of adenosine kinase mitigates oxidative and nitrosative stress in the retina of diabetic mice. Effect of AK inhibition on diabetes-induced oxidative and nitrosative stress in the retina. A) Oxidative stress measured by DHE staining. The fluorescence intensity indicates the level of superoxides production. The results represent the means ± SE of fold changes calculated using intensity, normalized to the level of the normal non-diabetic mice (n = 4–6). B) Nitrosative stress determined by immunofluorescence for nitrotyrosine. Scale bar: 20 μm.

Discussion

Biochemical studies have shown that inflammatory reactions (Joussen et al., 2004), including TNF-α release, are relatively early events that occur in response to diabetes before vascular dysfunction involving acellular capillary formation and neovascularization (Kern and Barber, 2008). Moreover, TNF-α has been shown to recruit leukocytes, cause vascular breakdown and promote neuronal injury at high levels (Joussen et al., 2009). Thus, treatments targeting early features of DR would provide long-term vascular benefits. Adenosine released at inflamed sites exhibits anti-inflammatory effects through A_{2A}AR (Bong et al., 1996). Although adenosine and its agonists are protective in animal models of inflammation, their therapeutic application has been limited by systemic side effects such as hypotension, bradycardia, and sedation (Williams, 1996). Moreover, adenosine usually disappears very rapidly in physiological or inflammatory conditions due to rapid reuptake and subsequent intracellular metabolism (Möser et al., 1989). The use of AK inhibitors represents one possible way to amplify the endogenous therapeutic effects of site- and event-specific accumulation of extracellular adenosine while minimizing hemodynamic toxicity.

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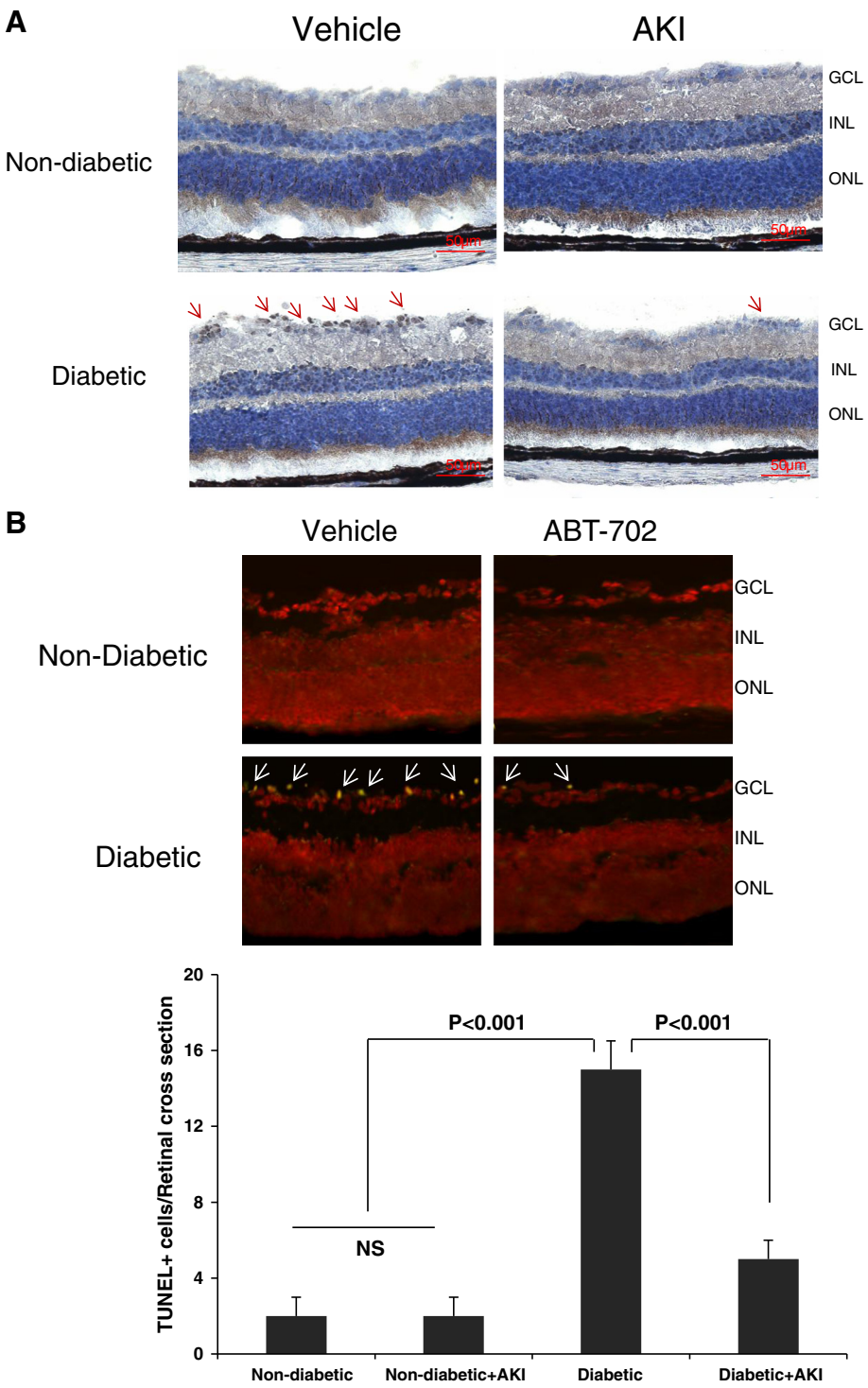

Fig. 7. Inhibition of adenosine kinase reduces retinal cell death in diabetic mice. A) Representative images with arrows show the localization of the apoptotic marker cleaved, activated caspase-3 in the ganglion cell layer in diabetic retina sections. ABT702 attenuated diabetes-induced cell death in the retina. B) Representative images with arrows showing the localization and quantitative analysis of TUNEL-positive cells in the ABT702-treated diabetic mice. The results represent the means ± SE of TUNEL-positive cells per retinal cross section (n = 4).

Endogenous adenosine levels in the brain are mainly dependent on the activity of AK, the key enzyme of adenosine metabolism (Gouder et al., 2004). This notion is based on several lines of evidence: 1) transgenic mice overexpressing AK are highly susceptible to stroke-induced brain injury (Pignataro et al., 2007); 2) pharmacological inhibition of AK provides seizure suppression in various models of epilepsy (Ugarkar et al., 2000); 3) inhibition of AK in hippocampal slices increases endogenous adenosine and depresses neuronal firing, whereas inhibition of adenosine deaminase has little or no influence (Huber et al., 2001); 4) AK activity is regulated in response to brain injury and is subject to developmental regulation (Studer et al., 2006; Pignataro et al., 2008). We demonstrated that AK has the same importance in the retina. In the present work, intraperitoneal injection of ABT 702 was found to cause a significant inhibition of ICAM-1 and TNF-α

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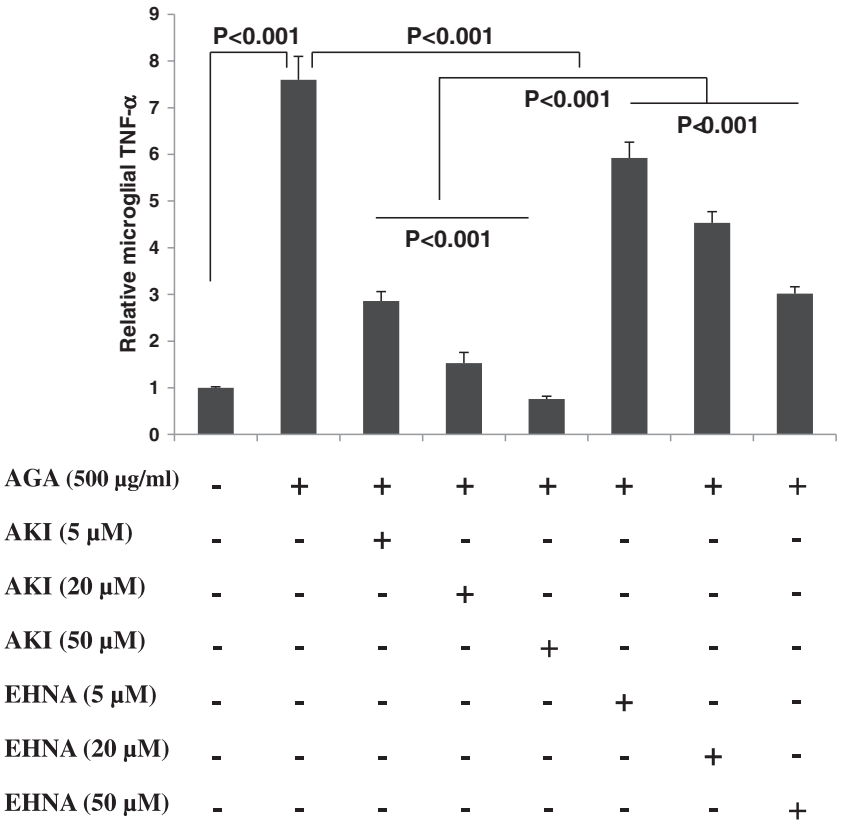

Fig. 8. Inhibition of adenosine kinase is more effective than adenosine deaminase in blocking TNF-α release in activated retinal microglial cells. Retinal microglial cells were treated with AGA (500 μg/mL, 16 h) in the presence of different doses of EHNA (ADA inhibitor), and ABT 702. TNF-α levels were determined by ELISA. Data shown are the mean ± SD of at least four different experiments.

mRNA as well as protein levels in the retina of diabetic mice, suggesting the curative effect of ABT 702 on inflammation associated with STZ-diabetic model. ABT 702 also prevented up-regulation of Iba1; supporting the hypothesis that ABT 702 reduces retinal inflammation through attenuation of microglia activation. Following this, we used primary culture of rat retinal microglial cells to gain insights into the

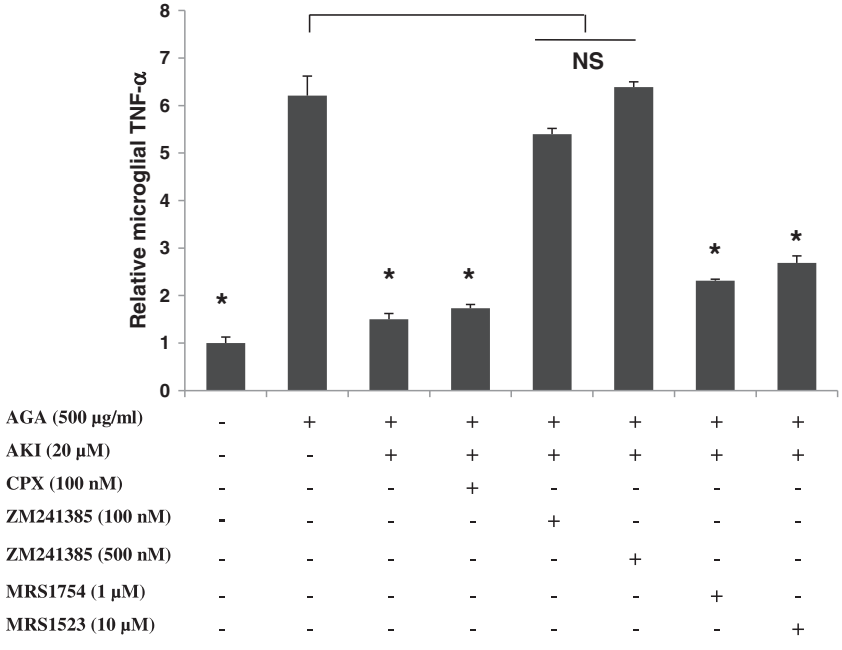

Fig. 9. Inhibition of adenosine kinase blocks TNF-α release via A_{2A}AR. Cells were treated with vehicle or ABT 702 (20 μM) 30 min before AGA treatment in the presence of subtype-selective AR antagonists for A_1AR (CPX, 100 nM), A_{2A}AR (ZM241385, 100 and 500 nM), A_{2B}AR (MRS 1754, 1 μM) and A_3AR (MRS 1523, 10 μM). *Significant compared to AGA treated microglial cells ($P < 0.05$).

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mechanism of ABT 702's anti-inflammatory effect. The results indicate that treatment of ABT 702 inhibited AGA-induced TNF-α release. Furthermore, ABT 702 was more effective than ADA inhibitor in inhibiting TNF-α release, suggesting a major role for AK in the regulation of extracellular adenosine.

The ability of ABT 702 to mitigate AGA-induced TNF-α release suggests the importance of inhibiting AK activity in ameliorating this inflammatory response through increasing adenosine levels. To test this hypothesis, the inhibitory effect of ABT 702 on AGA-induced TNF-α release was examined in the presence of AR subtype-selective antagonists in the retinal microglial cells. This inhibitory effect was successfully blocked only by 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-α][1,3,5]triazin-5-ylamino]ethyl} phenol (ZM 241385), a selective A_{2A}AR antagonist. These results suggest that ABT 702 inhibits AGA-induced TNF-α release in retinal microglia through A_{2A}AR. A_{2A}AR mediates the suppressive effects of adenosine in macrophages as well as microglial cells (Kreckler et al., 2006).

Diabetes or inflammation is associated with up-regulation of A_{2A}AR (Pang et al., 2010). High levels of A_{2A}ARs are found in macrophages and microglial cells that are poised, on activation, to abrogate the immune response (Trincavelli et al., 2008). In addition, hyperglycemia is associated with increased ENT1, possibly via an MAPK/ERK-dependent signaling pathway (Leung et al., 2005). ABT 702 inhibited the expression of both A_{2A}AR and ENT1 in the diabetic retina suggesting its ability to attenuate diabetic conditions. Further, we demonstrated that ABT 702 injection attenuated diabetes-induced reduction in AK expression. In the brain, AK expression is decreased following onset of injury thus potentiating the adenosine surge as a potential neuroprotective mechanism. Indeed, expression levels of AK might have a crucial role in determining the degree of brain injury (Li et al., 2008).

Next, we studied the effect of ABT 702 on oxidative and nitrosative stress in the retina in diabetes. In diabetes the retina experiences increased oxidative stress (Kowluru and Kanwar, 2007), and reactive oxygen species (ROS) are considered as a causal link between elevated glucose and the metabolic abnormalities important in the development of diabetic complications (Brownlee, 2001). ABT 702 decreased superoxides and nitrotyrosine levels in diabetic retina. The ability of ABT 702 to reduce inflammatory stress in the retina may rely on its inhibitory effect on oxidative and nitrosative stress.

Further, we studied the effect of ABT 702 on retinal cell death. Diabetes-induced retinal oxidative and nitrosative stress have been positively correlated with neuronal cell death (Asnaghi et al., 2003). Treating diabetic mice with ABT 702 blocked the increases in oxidative and nitrosative stress and significantly reduced cell death as revealed by decreased cleaved caspase-3 immunostaining and TUNEL assay in treated diabetic retinas. Neurons are highly susceptible to oxidative stress, which can induce apoptosis; therefore, it is likely that diabetes-induced oxidative stress leads to neuronal injury.

Finally, despite all the advantages for ABT 702 as a potential effective therapy for DR, given that the administration of ABT 702 by i.p. injection is invasive and stressful, oral administration of ABT 702 may be necessary but should be carefully developed (Kowaluk et al., 2000).

Conclusions

The data presented here provide experimental evidence that targeting AK can inhibit diabetes-induced retinal abnormalities that are postulated in the development of DR by potentially amplifying the endogenous therapeutic effects of site- and event-specific accumulation of extracellular adenosine. Thus, ABT 702 appears to be a useful therapy to possibly inhibit the development/progression of retinopathy, the sight threatening complication faced by diabetic patients.

Conflict of interest

We have no conflict of interests.

Acknowledgments

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Potential role of A_{2A} adenosine receptor in traumatic optic neuropathy

Saif Ahmad [a,b,*], Nadeem Fatteh [a], Nehal M. El-Sherbiny [a,c], Mohammad Naime [a,1], Ahmed S. Ibrahim [c], Ahmed M. El-Sherbini [a], Sally A. El-Shafey [a], Sohail Khan [d], Sadanand Fulzele [e], Joyce Gonzales [f], Gregory I. Liou [a,*]

[a] Department of Ophthalmology, Georgia Regents University (GRU), Augusta, GA, USA
[b] Departmet of Biological Sciences, College of Science and Arts, King Abdulaziz University, Rabigh, Saudi Arabia
[c] Department of Biochemistry, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt
[d] South Western Medical Center, Dallas, TX, USA
[e] Department of Orthopedics, Georgia Regents University (GRU), Augusta, GA, USA
[f] Vascular Biology Center, Georgia Regents University (GRU), Augusta, GA, USA

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ABSTRACT

In traumatic optic neuropathy (TON), apoptosis of retinal ganglion cells is closely related to the local production of reactive oxygen species and inflammatory mediators from activated microglial cells. Adenosine receptor A_{2A} (A_{2A}AR) has been shown to possess anti-inflammatory properties that have not been studied in TON. In the present study, we examined the role of A_{2A}AR in retinal complications associated with TON. Initial studies in wild-type mice revealed that treatment with the A_{2A}AR agonist resulted in marked decreases in the TON-induced microglial activation, retinal cell death and releases of reactive oxygen species and pro-inflammatory cytokines TNF-α and IL-6. To further assess the role of A_{2A}AR in TON, we studied the effects of A_{2A}AR ablation on the TON-induced retinal abnormalities. A_{2A}AR −/− mice with TON showed a significantly higher mRNA level of TNF-α, Iba1-1 in retinal tissue, and ICAM-1 expression in retinal sections compared with wild-type mice with TON. To explore a potential mechanism by which A_{2A}AR-signaling regulates inflammation in TON, we performed additional studies using hypoxia- or LPS-treated microglial cells as an in vitro model for TON. Activation of A_{2A}AR attenuates hypoxia or LPS-induced TNF-α release and significantly repressed the inflammatory signaling, ERK in the activated microglia. Collectively, this work provides pharmacological and genetic evidence for A_{2A}AR signaling as a control point of cell death in TON and suggests that the retinal protective effect of A_{2A}AR is mediated by attenuating the inflammatory response that occurs in microglia via interaction with MAPKinase pathway.

1. Introduction

Traumatic optic nerve injury is commonly seen in motor vehicle accidents, assaults, war and in the natural disaster. Traumatic optic nerve injury is usually the consequence of a severe blunt head trauma, often a frontal blow severe enough to cause loss of consciousness. Unfortunately, there are currently no proven treatments that can prevent the damage associated with an acute traumatic optic neuropathy (TON). Prognosis for the recovery of vision in TON is still poor, nevertheless, animal models for TON are often used, mostly because they are easy to perform and can be well standardized (Levkovitch-Verbin, 2004). Retinal ganglion cell (RGC) death is known to be a fundamental pathological process in traumatic optic injury including TON. Several common mechanisms have been hypothesized to underlie apoptotic processes, including interruption of trophic support, oxidative stress, and increased extracellular glutamate levels that result in excitotoxicity. These stimuli associated with the injured RGCs often activate retinal microglia, which release pro-inflammatory cytokines and cytotoxic molecules to further exacerbate the degenerative process (Kreutzberg, 1996). These findings suggest that pharmacological interventions that reduce inflammation may be effective neuroprotectants for TON.

Under stress and ischemic conditions, the local tissue concentration of extracellular adenosine is increased due to its synthesis from the released ATP. This nucleoside has been proposed to modulate a variety of physiological responses by stimulating specific adenosine receptors (AR), which are classified as A1, A2A, A2B, and A3 subtypes (Collis and Hourani, 1993). These receptors can be distinguished based on their affinities for adenosine agonists and antagonists. In addition, these receptors are classified based on their mechanism of signal

Abbreviations: TON, traumatic optic neuropathy; TNF-α, tumor necrosis factor-α; ELISA, Enzyme-linked immunosorbent assay; ROS, reactive oxygen species; MAP kinase, Mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; AR, adenosine receptor; CGS21680, 2-p-[2-Carboxyethyl]phenethylamino-5′-N-ethylcarboxamidoadenosine; LPS, lipopolysaccharides; NECA, 5′-*N*-Ethylcarboxamidoadenosine; ZM241385, 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol.

* Corresponding authors at: Department of Ophthalmology, Georgia Regents University, 1120 15th Street, Augusta, GA 30912, USA. Tel.: +1 706 721 4599; fax: +1 706 721 1158.
E-mail addresses: sahmad@gru.edu, asaif77@yahoo.com (S. Ahmad), giliou@gru.edu (G.I. Liou).
[1] Biochemistry Lab, Regional Research Institute of Unani Medicine, CCRUM, Srinagar, J&K-190006, India.

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Table 1
The primer sets used for the detection of mouse genes by quantitative real-time PCR analysis.

Gene	Primer sequence (5′–3′)	Accession number
TNF-α	CCCTCACACTCAGATCATCTTCT GTCACGACGTGGGCTACAG	NM_013693.2
ICAM-1	CGCTGTGCTTTGAGAACTGTG ATACACGGTGATGGTAGCGGA	NM_010493
Iba-1	GTCCTTGAAGCGAATGCTGG CATTCTCAAGATGGCAGATC	NM_019467
A_2aAR	TCCACTCCGGTACAATGGCTTGGT AGCATGGGGGTCAGGCCGAT	NM_009630.2
Mice IL-6	TAGTCCTTCCTACCCCAATTTCC TTGGTCCTTAGCCACTCCTTC	NM_031168.1
GAPDH	CAT GGC CTC CAA GGA GTAAGA GAG GGA GAT GCT CAG TGT TGG	M32599
18S	AGT GCG GGT CAT AAG CTT GC GGG CCT CAC TAA ACC ATC CA	NR_003278

transduction. A1 and A3 receptors interact with pertussis toxin-sensitive G proteins of the Gi and Go family to inhibit adenylate cyclase. The A2A receptor stimulates adenylate cyclase through Gs coupling (Fredholm et al., 1994). A2B receptor stimulates phospholipase C activity through Gq (Feoktistov et al., 1999). The increased adenosine at inflamed sites exhibits anti-inflammatory effects to protect against cellular damage through A_{2A}AR (Bong et al., 1996; Ralevic and Burnstock, 1998; Ohta and Sitkovsky, 2001). A_{2A}AR agonist treatment blocks the inflammation, and functional and histological changes associated with diabetic nephropathy in wild-type diabetic mice but not in the A_{2A}AR −/− diabetic mice (Awad et al., 2006). We found that treatment with the A_{2A}AR agonist resulted in marked decreases in diabetes-induced retinal cell death and TNF-α release (Ibrahim et al., 2011a). We also found that activation of A_{2A}AR in the stressed retinal microglial cells was the most efficient in mediating TNF-α inhibition (Liou et al., 2008). Furthermore, our work showed that diabetic A_{2A}AR −/− mice had significantly more TUNEL-positive cells, TNF-α release, and ICAM-1 expression compared with diabetic wild-type mice (Ibrahim et al., 2011a). The proposed mechanism of chronic retinal injury in diabetic retinopathy is RGC death associated with activation of an inflammatory pathway and an anti-inflammatory pathway involving A_{2A}AR signaling. We propose that A_{2A}AR signaling may also play a similar role in the acute treatment of TON.

Recent efforts to understand how neurotoxic inflammatory cytokines are produced have shown that MAPKinase signaling pathway is one of the attractive targets for intervention in human inflammatory-associated diseases such as diabetes. However, this pathway does not operate alone, but rather interacts with other signaling systems, such as Gs-coupled receptor transducing pathway. Activation of this pathway results in accumulation of cAMP that interacts with the MAPKinase signaling pathway to regulate cell functions (Gerits et al., 2008).

Previously we demonstrated the anti-inflammatory effect of A_{2A}AR in acute (Liou et al., 2008) and chronic (Ibrahim et al., 2011a) retinal

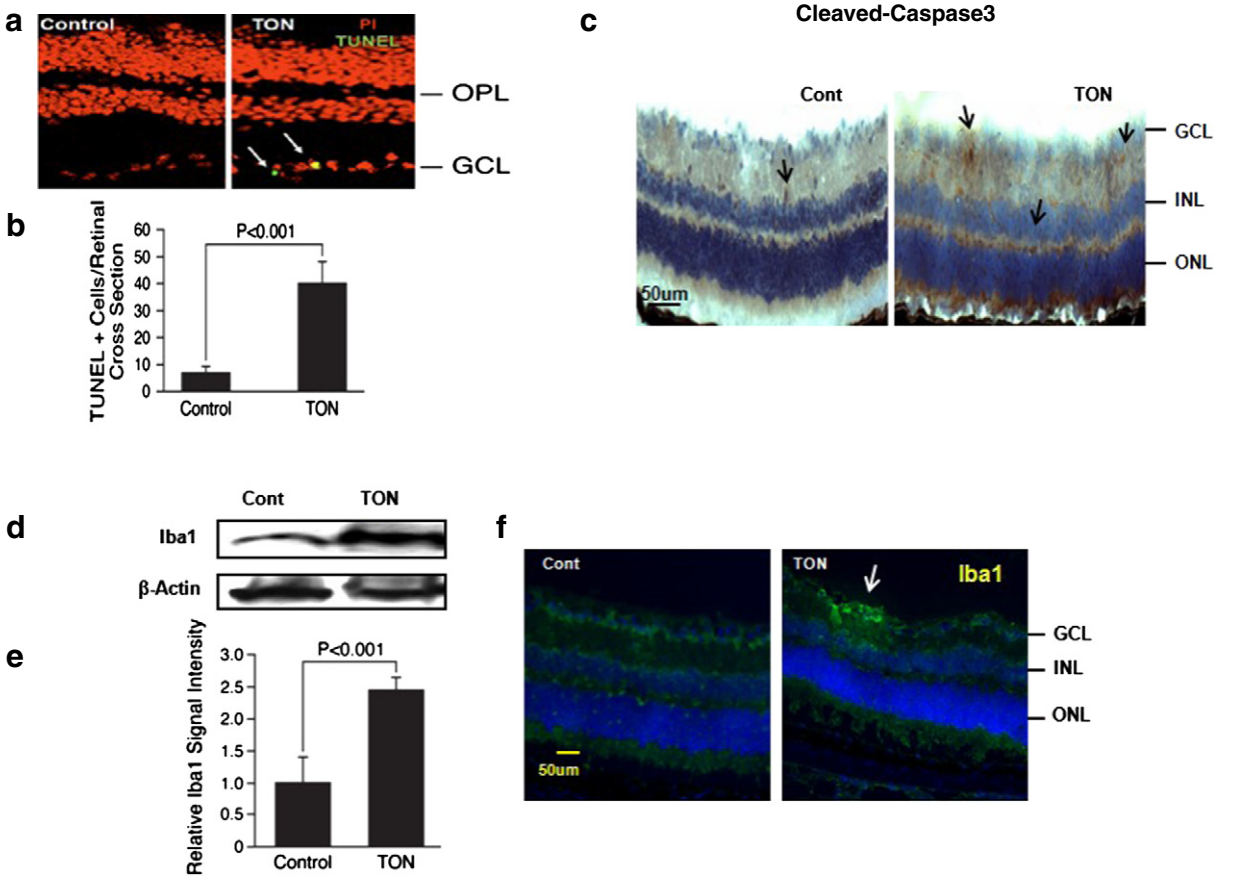


Fig. 1. TUNEL assay, cleaved-caspase3 activation and Iba1 expression on the retina of the mouse model of TON. a–b) Retinal distribution of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells identified in TON and control. Sections were counterstained with propidium iodide (PI). c) Quantitative analysis of TUNEL-positive cells in the retinal cross section of TON and control. TUNEL+ cells were counted in 10 adjacent locations along the vertical meridian within 4 mm of the optic disk (10 fields/retina sections). c) Immunohistochemical analysis of activated caspase3 in retinal section of TON vs. control. DAB (3, 3′-diaminobenzidine) produces a dark brown reaction product of cleaved caspase3. d) Immunoblotting analysis of microglial activation marker Iba1 expression in TON vs. control in the retina. e) Densitometry analysis of Iba1 and actin ratio by Image J software, NIH. f) Immunolabeling of Iba1 (green) with nuclear marker, DAPI (blue) in retinal section of TON vs. control. Data shown are the mean ± SD (n = 4). *P < 0.01, **P < 0.001, ***P < 0.0001.

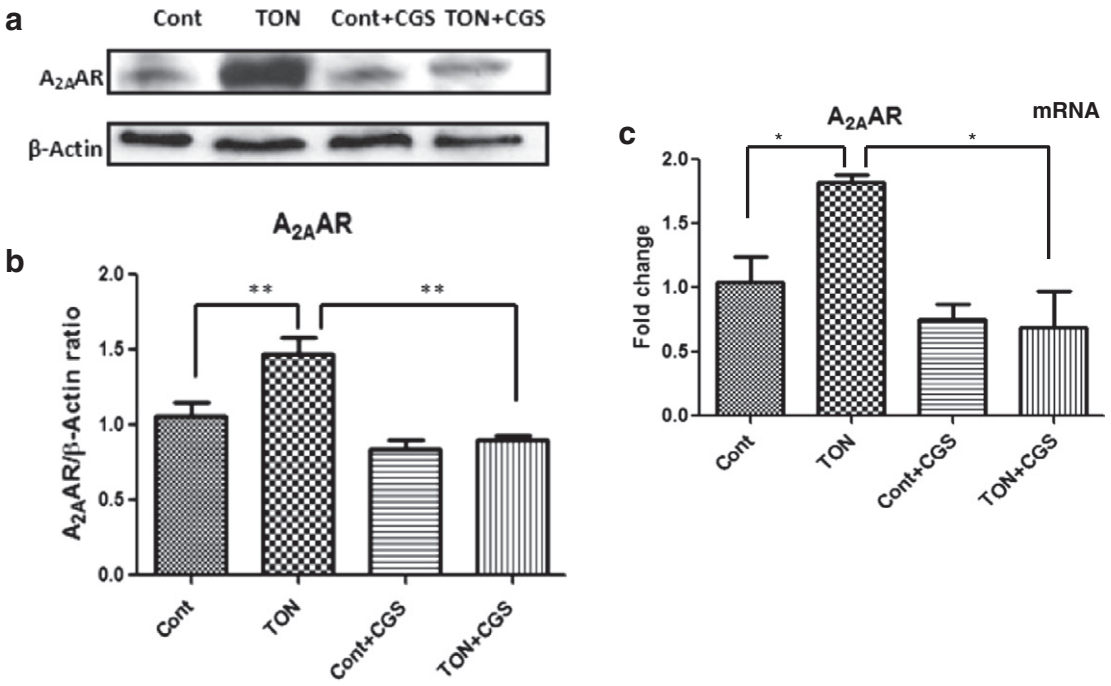


Fig. 2. Effect of $A_{2A}AR$ agonist treatment on $A_{2A}AR$ expression in the mouse model of TON. a–b) Western blot analysis of $A_{2A}AR$ protein expression in the retinal tissue in TON vs. control, with and without CGS21680 treatment. Densitometry analysis was done for $A_{2A}AR$ and actin ratio by Image J software. c) Retinal $A_{2A}AR$ mRNA was determined by real-time PCR in TON vs. control, with and without CGS effect. Data shown are the mean ± SD (n = 4). *P < 0.01, **P < 0.001, ***P < 0.0001.

inflammation. Currently, we seek to determine the contribution of $A_{2A}AR$ in retinal protection against TON-induced retinal inflammation and injury. Moreover, we pursue to gain insight into the underlying signaling involved therein. Here, we report evidence that activation of $A_{2A}AR$ plays a protective role in TON-induced retinal cell death by enhancing the anti-inflammatory signaling including the interaction with MAPKinase pathway. These findings suggest that $A_{2A}AR$ agonists might be promising innovative agents in the treatment of TON.

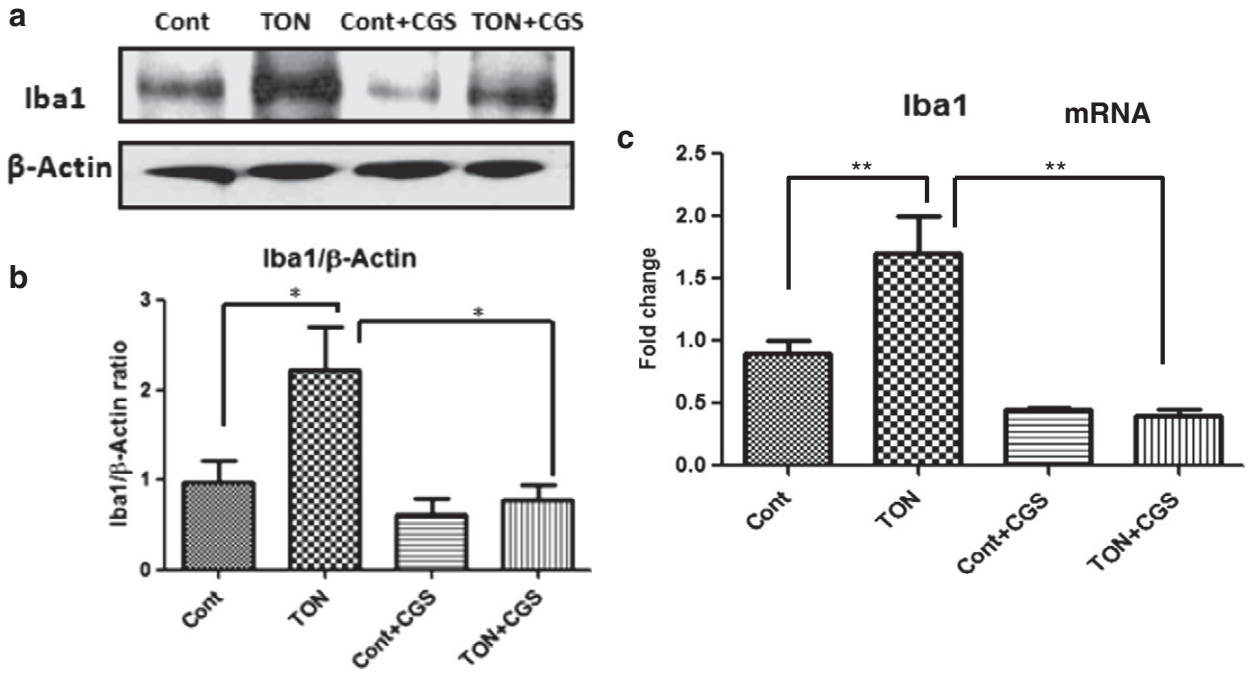


Fig. 3. Effect of $A_{2A}AR$ agonist treatment on the retinal microglial activity in the mouse model of TON. a–b) Analyses of Iba1-expressing retinal cells in TON model with and without agonist treatment by Western blot. Densitometry analysis was done for Iba-1 and actin ratio by Image J software. c) Retinal Iba1 mRNA was determined by real-time PCR in TON vs. control, with and without CGS effect. Data shown are the mean ± SD (n = 4). *P < 0.01, **P < 0.001, ***P < 0.0001.

2. Materials and methods

2.1. Animal preparation and experimental design

All procedures with animals were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare publication, NIH 80-23) and Georgia Health Sciences University guidelines. Eight-week-old male $A_{2A}AR-/-$ and corresponding littermate controls, wild-type (WT) mice in C57BL/6 background, were matched according to sex, age, and weight. $A_{2A}AR-/-$ mice (Chen et al., 1999) were a kind gift from Dr. J F Chen, Harvard Medical School, Boston, MA. Mice were anesthetized according to standard protocol and limbal conjunctival peritomy was performed on one eye of each mouse. Forceps dissection under the conjunctiva posteriorly allowed access to the optic nerve, upon which pressure was placed 1 mm posterior to the globe until pupillary dilation was noted (approximately 10 s). Blood vessel close to optic nerve was carefully avoided in TON surgery. Mock-operated contralateral eye served as the control. After one week, all mice were sacrificed. Eyes were enucleated and sectioned for immunohistochemistry. Retinas were harvested for Western or Real Time PCR analysis. In pharmacologic studies, age-, weight- and sex-matched C57BL/6 mice were rendered optic nerve crush and then injected i.p. with vehicle (DMSO), or CGS21680 (25 μg/kg) every other day for the duration of the study (n = 4–6/group) (Genovese et al., 2009).

2.2. Terminal dUTP nick end-labeling (TUNEL)

TUNEL was performed in frozen sections using the TACS-2 TdT Fluorescein In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD) counter-stained with propidium iodide, according to the manufacturer's suggestions. Briefly, sections were hydrated with alcohol 100%, 95%, and 70%, then fixed in 3.7% paraformaldehyde. After washing, slides were incubated in mixture of TdT, Mn^{+2}, and TdT dNTP for 1 h at 37 °C. The reaction was stopped with TdT Stop Buffer for 5 min. After washing with deionized water, the slides were incubated with Streptavidin–HRP (diluted 1:200) solution for 20 min at room temperature. Slides were counter-stained, mounted, covered with coverslips and visualized by a confocal microscopy (LSM 510, Carl Zeiss, Inc.). Apoptotic cells were identified as doubly labeled with TdT Fluorescein and propidium iodide and only nuclei that were clearly labeled yellow were scored.

2.3. Immunohistochemical analysis

Immunofluorescence analysis was performed using frozen retinal sections. Briefly, cryostat sections (15 μm) were fixed in 4% paraformaldehyde, blocked with 10% normal goat serum (NGS) and then incubated overnight at 4 °C with primary antibodies: rabbit anti-Iba-1 (Wako Pure Chemical, Wako, TX), or mouse anti-ICAM-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) together with Texas Red-labeled Isolectin B4 (Invitrogen, Carlsbad, CA), anti-gp91phox (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-GFAP (Dako, USA) with FITC green, rabbit anti-A2aAR (Abcam, USA), and rabbit anti-cleaved Casape3 (Cell Signaling, USA). Thereafter, sections were briefly washed with PBS and incubated with appropriate secondary antibodies. Slides were examined by confocal microscopy (LSM 510, Carl Zeiss). Specificity of the reaction was confirmed by omitting the primary antibody. Data (10 fields/retina, $n = 4–6$ in each group) were analyzed using fluorescence microscopy and Ultra-View morphometric software or Image J software (NIH) to quantify the intensity of immunostaining.

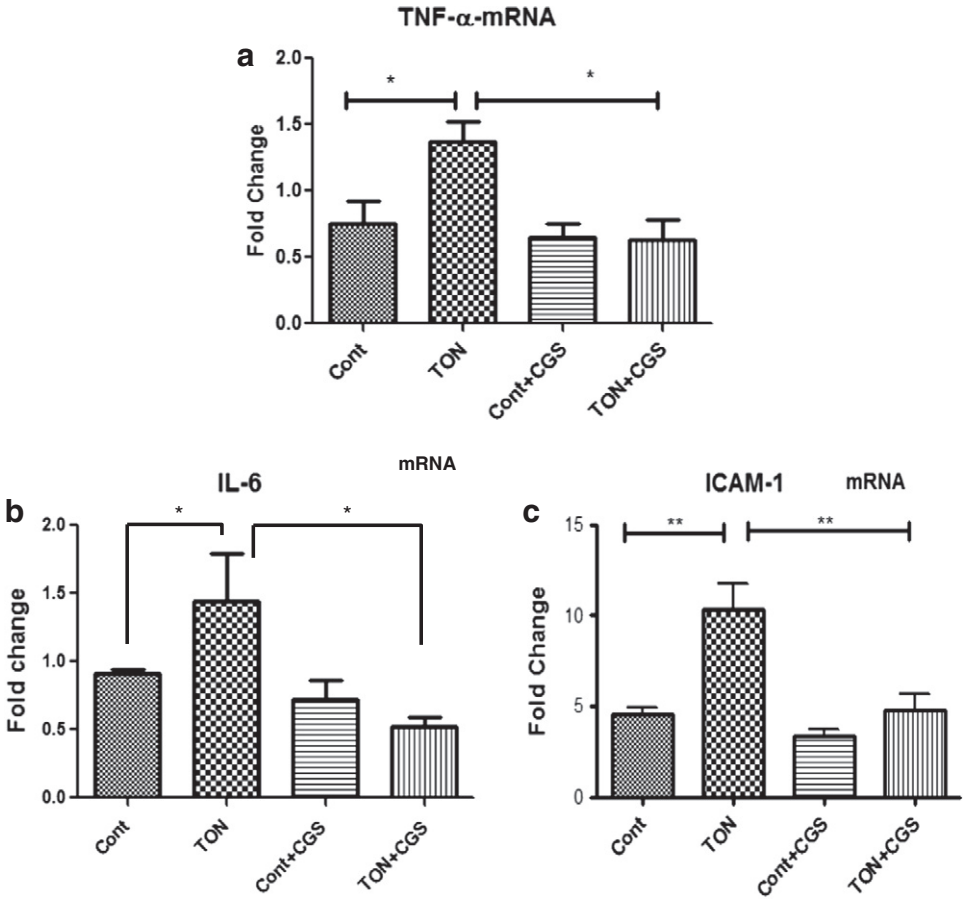
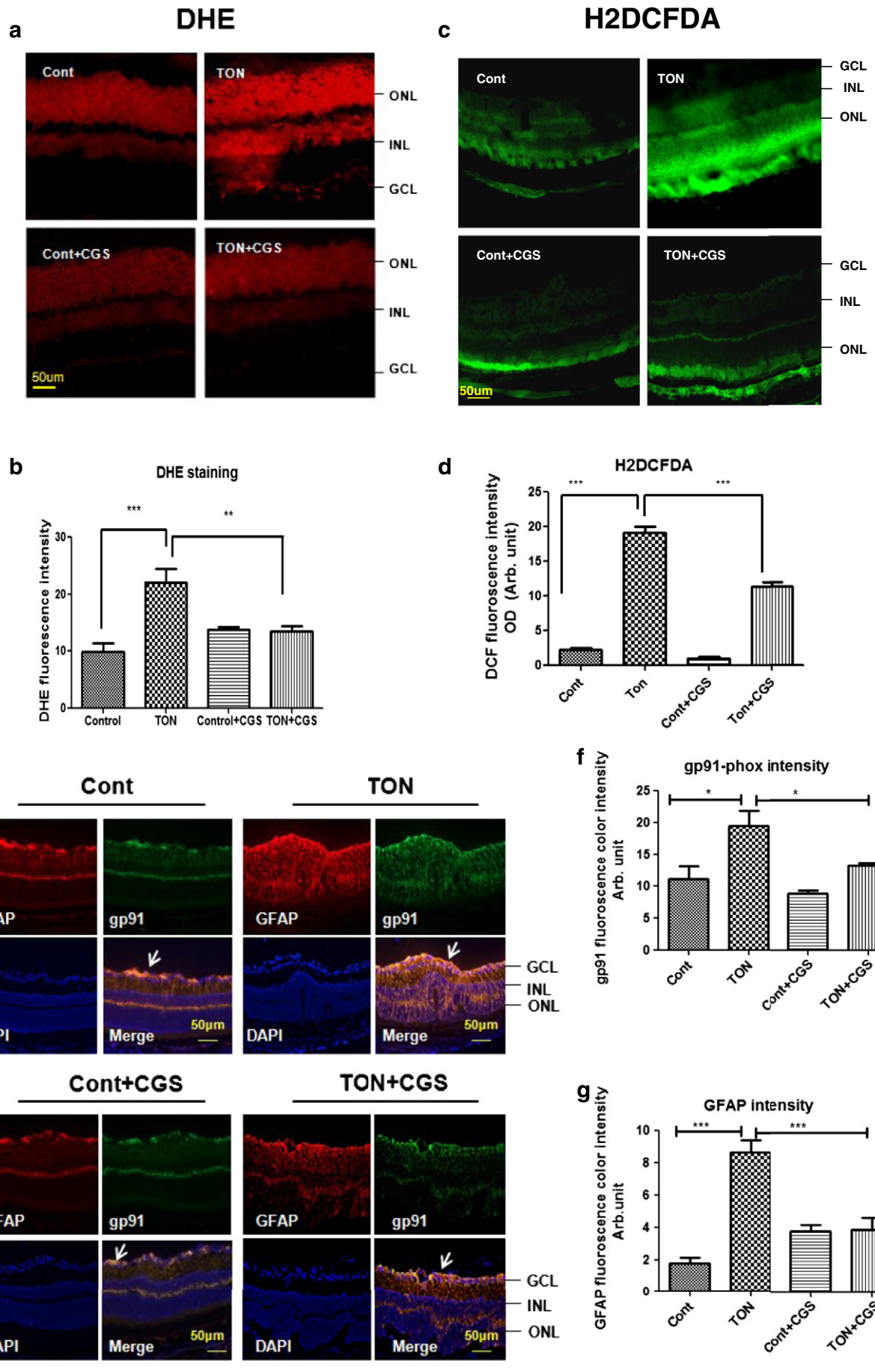


Fig. 4. Effect of $A_{2A}AR$ agonist treatment on the retinal expression of pro-inflammatory cytokines in the mouse model of TON. a–c) Real-time PCR analysis of TNF-α, IL-6 and ICAM-1 mRNA expression in the retina of TON, with and without agonist. Data shown are the mean ± SD (n = 4). *P < 0.01, **P < 0.001, ***P < 0.0001.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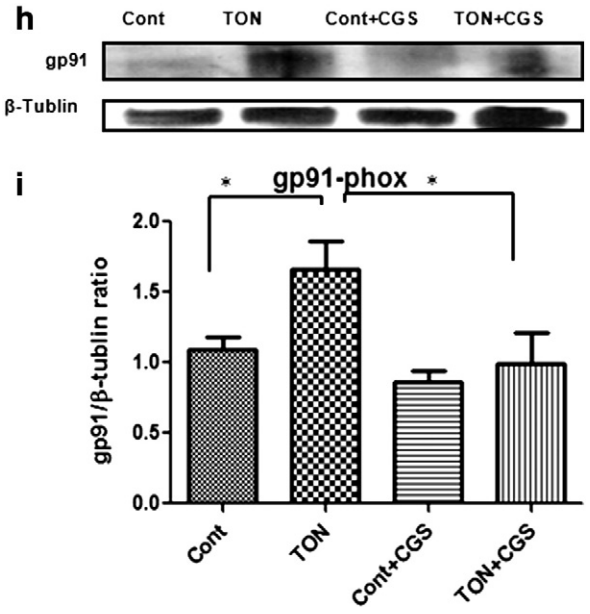

Fig. 5. Effect of A_{2A}AR agonist treatment on the retinal levels of oxidative stress in the mouse model of TON. a–b) Superoxide levels determined by dihydroethidium (DHE) fluorescence. Fluorescence intensities were quantified by Image J software, NIH. c–d) ROS activities determined by 2,7-dichlorodihydro-fluorescein diacetate (H2DCFDA). Fluorescence intensities were quantified by Image J software, NIH. Data shown are the mean ± SD (n = 4–5). e–g) Immunofluorescence analysis of NADPH oxidase subunit gp91-phox expression in retina. Gp91-phox (green), GFAP (red) with DAPI (blue). Fluorescence intensities of gp91 and GFAP were quantified by Image J software, NIH. h–i) Retinal gp91-phox protein expression was determined by Western blotting in TON vs. control with and without agonist. Densitometry analysis of gp91 and tublin band ratio was done by Image J software, NIH. Data shown are the mean ± SD (n = 4). GCL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer. *P < 0.01, **P < 0.001, ***P < 0.0001.

Immunohistochemistry analysis was done by MOM kit (Vector Laboratories, USA). Protocol was used according to Kit.

2.4. Analysis of dihydroethidium (DHE) fluorescence for the detection of superoxide

The detection of superoxide anion in the mouse eye sections was performed as described previously (Inaba et al., 2009). In brief, mouse eyes were frozen in OCT and stored at −80 °C until use. Enzymatically intact eye sections were thawed in room temperature, rehydrated with PBS, incubated with dihydroethidium (DHE; 10 μMol/L in PBS) for 30 min at 37 °C in a humidified chamber protected from light. After incubation, sections were washed with PBS. DHE is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus and emits red fluorescence. For the detection of ethidium, samples were examined with a fluorescence microscope (Axioskop 2 plus with AxioCam; Carl Zeiss, Germany; Excitation/Emission wavelengths: 518/605 nm). DHE fluorescence was quantified using Image J software (NIH).

2.5. Measurement of ROS activity by H2DCFDA for oxidative stress

Dichlorofluorescein (DCF), the oxidation product of 2,7-dichlorodihydro-fluorescein diacetate (H2DCFDA; Invitrogen) emits a green fluorescent signal and is a marker of cellular oxidation by reactive oxygen species (ROS) including hydrogen peroxide, peroxynitrite, and hydroxy radicals (Al-Shabrawey et al., 2008). Retinal sections were incubated with H2DCFDA (10 μM) in HBSS buffer for 20 min at 37 °C. DCF formation was measured with fluorescence microscopy to collect the images, and with computer-assisted morphometry to determine the fluorescence intensity.

2.6. Protein extraction and Western blot analysis

Washed cultured cells and retinal tissue were lysed in modified RIPA buffer (Upstate, Lake Placid, NY), containing 50 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1% Nonidet P-40, 0.25% deoxycholate, supplemented with 40 mmol/L NaF, 2 mmol/L Na_3VO_4, 0.5 mmol/L phenylmethylsulfonyl fluoride and 1:100 (v/v) of proteinase inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation at 12,000 ×*g* at 4 °C for 30 min. Protein was determined by DC Protein Assay (Bio-Rad, Hercules, CA) and 50–100 μg was boiled in Laemmli sample buffer, separated by SDS-PAGE on a gradient gel (4 to 20%) (Pierce, Rockford, IL), transferred to nitrocellulose membrane and incubated with specific antibodies. Antibodies for β-actin (Sigma), Iba1, A_{2A}AR, phospho-ERK and ERK (Cell Signaling Technology, Beverly, MA) were detected with a horseradish peroxidase-conjugated antibody and ECL chemiluminescence (Amersham BioSciences, Buckinghamshire, UK). Intensity of immunoreactivity was measured by densitometry.

2.7. Isolation of RNA, synthesis of cDNA, and real-time PCR

Total RNA was isolated from mouse retina using SV Total RNA Isolation kit (Promega, Madison, WI) following manufacturer's instructions, and the quality of the RNA preparations was monitored by absorbance at 260 and 280 nm (Helios-Gamma, Thermo Spectronic, Rochester, NY). The RNA was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using iScript reagents from Bio-Rad in a programmable thermal cycler (PCR-Sprint, Thermo Electron, Milford, MA). Fifty ng of cDNA was amplified in each real-time PCR using Bio-Rad iCycler, ABgene reagents (Fisher scientific) and appropriate primers. Average of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA was used as the internal control for normalization (Table 1).

2.8. Primary retinal microglial culture

Microglial cells were isolated from retinas of newborn SD rats according to a previous procedure (Wang et al., 2005) with minor modifications. Briefly, retinas were dissected from newborn SD rat pups. Tissues were collected into 0.01 M PBS and washed with ice-cold 0.01 M PBS, digested with 0.125% trypsin for 3–5 min and mixed with DMEM/F12 (1:1) (Invitrogen, CA) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, GA) and 1% penicillin/streptomycin (Mediatech, Herndon, VA). Retina pieces were triturated by passing them through a disposable pipette several times until cells were dispersed. Cells were then filtered through a mesh (100 μm), collected by centrifugation, resuspended in the culture medium and plated onto T150 cell culture flasks (Corning, NY) at a density of 2×10^5 cells/cm^2. All cultures were maintained in a humidified CO_2 incubator at 37 °C and 5% CO_2 and fed on the third day, then once every 4 days. After 2 weeks, microglial cells were harvested by shaking the flasks at 100 rpm for 1 h. The cell suspension was centrifuged and detached cells were replated in DMEM/F12 (1:1) + 10% FBS overnight and then in serum-free/low protein media (Cellgro Complete containing 0.1% bovine serum albumin; Mediatech, Manassas, VA) at designated densities for various experiments. The purity of microglial cultures was 98%, as determined by immunocytochemical staining analysis and by cytometry (Ibrahim et al., 2011a) for Iba1, a microglial marker. The morphology of microglia in culture was carefully examined by phase-contrast and fluorescence microscopy.

2.9. Drug treatment effects on cultured microglial cells

Microglial cells were seeded at a density of 5×10^5 cells/well in 24-well tissue culture plate, or 1×10^5 cells/well in 96-well plate. One day after seeding, the wells were washed with Cellgro Complete (Mediatech, Manassas, VA) and incubated in the same media with various treatments. Cells were pretreated with A_{2A}AR-selective agonist (CGS21680, Tocris, Ellisville, MO, 20 μM) or A_{2A}AR-selective antagonist (ZM 241385, Tocris, Ellisville, MO, 2 μM) at the indicated concentrations reported previously or vehicle dimethylsulfoxide (DMSO) for 30 min at 37 °C before hypoxia or LPS treatment (30 ng/ml). NECA (2 μM) was used as nonselective AR agonist. Microglial cells were placed in 1% oxygen (hypoxic) and room air (normoxic) conditions at 37 °C for the indicated time. Both groups were then placed under normoxic conditions for 24 h. At indicated time points, cells were homogenized for Western blot analysis and culture media were taken and analyzed for TNF-α by ELISA.

2.10. Enzyme-linked immunosorbent assay (ELISA) for TNF-α

TNF-α levels in the supernatants of culture media were estimated with ELISA kits (R&D, Minneapolis, MN) per the manufacturer's instructions. Standards and samples were added and bound by the immobilized antibody. After washing, an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells followed by a substrate solution yielding a colored product. The intensity of the color was measured at 450 nm. The sample levels were calculated from the standard curve and corrected for protein concentration.

2.11. Statistics

The results are expressed as mean ± SD. Differences among experimental groups were evaluated by analysis of variance (one-way ANOVA), and the significance of differences between groups was assessed by the posthoc test (Newman–Keuls multiple comparison). Significance was defined as $P < 0.05$. Data were analyzed by GraphPad PRISM software.

3. Results

3.1. TON-induced microglial activation and retinal cell death

Because activation of the A_{2A}AR has been implicated in the anti-inflammatory actions of adenosine in experimental diabetes (Ibrahim et al., 2011a), we hypothesized that this receptor may also be effective in protecting retinal neurons from TON-induced inflammation and neurotoxicity. Quantitative analysis of TUNEL-FITC and propidium iodide-double labeled cells in retinal tissue showed a significant increase in the frequency of retinal cell death mostly in the ganglion cell layer 7 days after optic nerve crush (Fig. 1a & b). That the retinal cell death was through apoptosis was confirmed with activated caspase-3 by immunohistochemistry (Fig. 1c). Following this, we addressed an interesting feature, acquisition of reactive microglial phenotype that could be an important determinant for understanding the mechanisms by which optic nerve crush induces RGC death. In this regard, we noted that when microglia encountered TON milieu, they became activated as indicated by increased Iba-1 expression (Fig. 1d, e & f).

3.2. Role of A_{2A}AR agonist in microglial activation and A_{2A}AR expression

To test this hypothesis, we treated mice with the procedures that crushed optic nerve in one eye. Treated mice were further treated with the A_{2A}AR-selective agonist, CGS21680, or with vehicle via i.p. injection every two days. At day 7, retinal microglial activation, retinal cell death, pro-inflammatory cytokine release, and ROS production were compared. We determined the effect of optic nerve crush on the expression of A_{2A}AR in the retina. Compared with the contralateral eyes, the eyes with crushed optic nerves demonstrated a significant increase in the levels of A_{2A}AR protein expression (Fig. 2a & b). The treatment with CGS21680 resulted in a marked reduction of TON-associated A_{2A}AR protein and mRNA up-regulation (Fig. 2c). These results demonstrated that under TON-associated stress, a self-defense system including A_{2A}AR assumes a compensatory effect. However, CGS21680 treatment in the eyes with crushed optic nerves significantly reduced Iba-1 protein and mRNA expression (Fig. 3a–c), suggesting reduced microglial cell activity.

3.3. Role of A_{2A}AR in retinal inflammation

We next determined the effect of optic nerve crush on the levels of pro-inflammatory cytokines in the retina. Compared with the contralateral eyes, the eyes with crushed optic nerves demonstrated a significant increase in the levels of cytokines (TNF-α and IL-6) along with intercellular adhesion molecule, ICAM-1. CGS21680 treatment in the eyes with crushed optic nerves significantly reduced TON-associated cytokines and ICAM-1 mRNA expression (Fig. 4a–c).

3.4. Role of A_{2A}AR in retinal oxidative stress

We then determined the effect of optic nerve crush on the levels of oxidative stress in the retina by two methods (DHE and DCF) that measure superoxide generation and different ROS. Compared with the contralateral eyes, the eyes with crushed optic nerves demonstrated a significant increase in the levels of all the ROS determined (Fig. 5). The treatment with CGS21680 resulted in a marked reduction of TON-associated ROS release (Fig. 5a–d). As the report suggests that NADPH oxidase is the main source of free radical generation (Al-Shabrawey et al., 2008; Brennan et al., 2009), we checked the expression level of gp91phox (NADPH oxidase subunit) and GFAP by immunofluorescence and Western blotting. The expression level of gp91phox and GFAP was significantly high in TON compared with the control eye and CGS21680 treatment significantly modulated the NADPH oxidase and GFAP activation.

3.5. Role of inflammation in A_{2A}AR −/− mice

To further assess the role of A_{2A}AR in TON, we studied the effects of A_{2A}AR ablation on TON-induced retinal inflammation. As shown in Fig. 6a, mRNA expression of TNF-α in the retinas of TON-associated A_{2A}AR −/− mice were notably increased with their age-matched TON-associated WT control mice. Furthermore, Iba1 mRNA in the A_{2A}AR −/− mice retina was significantly increased (~1.5-fold) compared with WT (Fig. 6b). Immunofluroscence analysis of ICAM-1 expression in retinal section showed marked increase in A_{2A}AR −/− retina compared with WT littermates with TON (Fig. 6c). Taken together, these results suggest that A_{2A}AR plays a crucial role in limiting retinal inflammation and neuronal cell injury associated with TON.

3.6. Activation of A_{2A}AR attenuates hypoxia- or LPS- induced TNF-α release in retinal microglial cells

After having shown that A_{2A}AR −/− mice with TON exhibit profound retinal cell death and inflammation, we next sought to explore a potential mechanism by which A_{2A}AR signaling regulates inflammation in TON. To do this, additional studies using microglial cells treated with hypoxia or LPS were performed. Treatment of microglia with hypoxia has been shown to simulate inflammation (Wang et al., 2005; Quan et al., 2007; Wang et al., 2007). As shown in Fig. 7b, treatment of retinal microglial cells with hypoxia, but not with normoxia, triggered a prominent increase in TNF-α release. To determine the role of the A_{2A}AR in regulating TNF-α release, we first examined the effect of the selective A_{2A}AR agonist, CGS21680, in hypoxia- or LPS-induced TNF-α release. As shown in Fig. 7f & g, activation of A_{2A}AR with CGS21680 inhibited TNF-α release. To confirm this point, we used

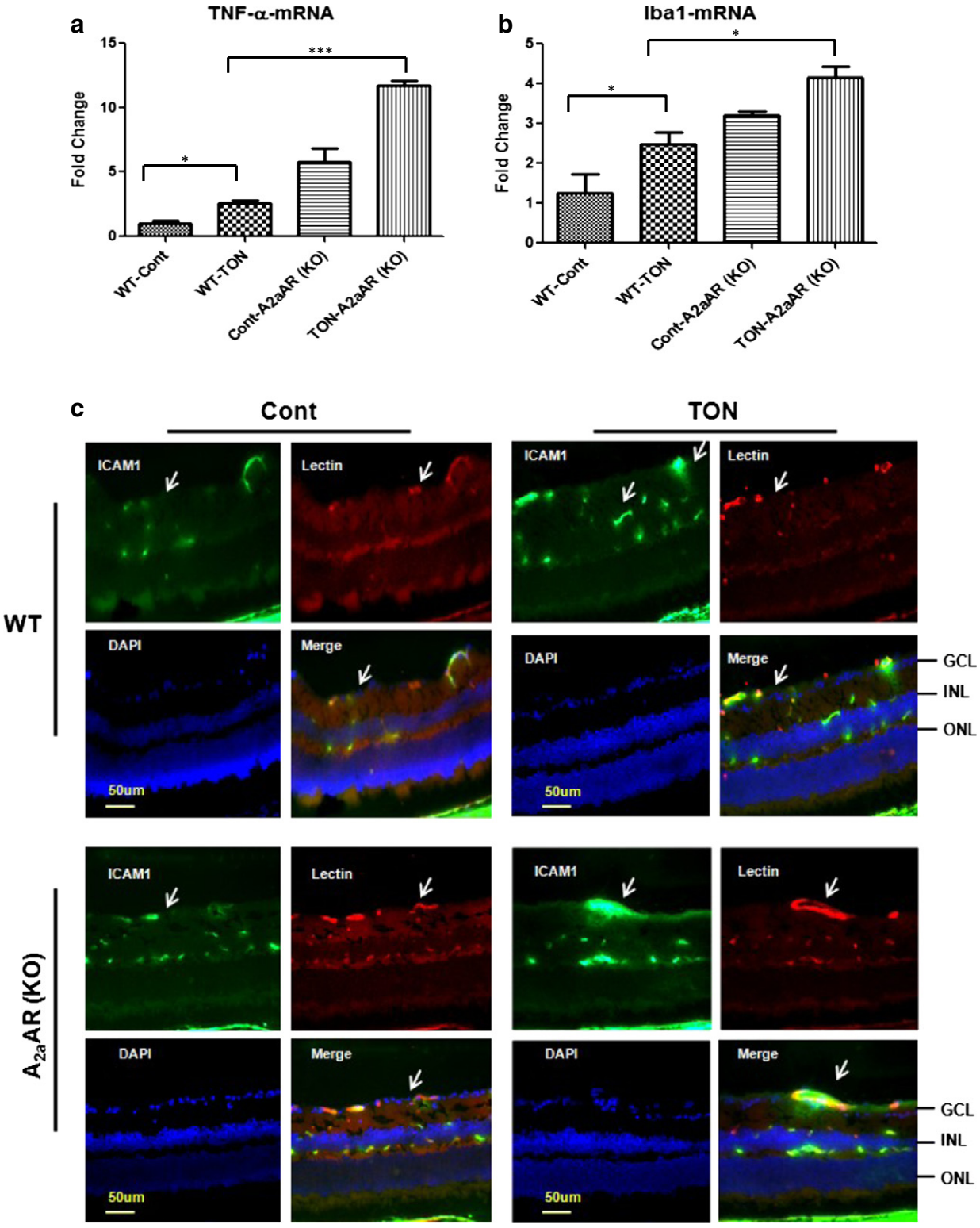

Fig. 6. Effect of A_{2A}AR depletion on the retinal mRNA expression of TNF-α, Iba1 and ICAM-1 in the mouse model of TON. a–b) Real-time PCR analysis of Iba1 and TNF-α expression, WT vs. A_{2A}AR-KO mice. c) Immunofluorescence analysis of intercellular adhesion molecule 1 (ICAM-1) distribution in the retinal sections, WT vs. A_{2A}AR-KO. Sections were stained with ICAM1 (green), endothelial cells marker Isolectin B4 (red) and DAPI (blue). Data shown are the mean ± SD (n = 4). *P < 0.01, **P < 0.001, ***P < 0.0001.

A_{2A}AR non-selective agonist (NECA) and A_{2A}AR selective antagonist (ZM241385) to treat the microglial cells followed with LPS treatment. We showed that LPS significantly elevated TNF-α release as compared with control. ZM241385 treatment alone did not alter the TNF-α release. NECA treatment significantly decreased TNF-α level. LPS + NECA + ZM treatment reversed the effect of TNF-α release by LPS + NECA, suggesting that A2AAR is responsible for the reduction of TNF-α release. Together, these findings identify a signaling through

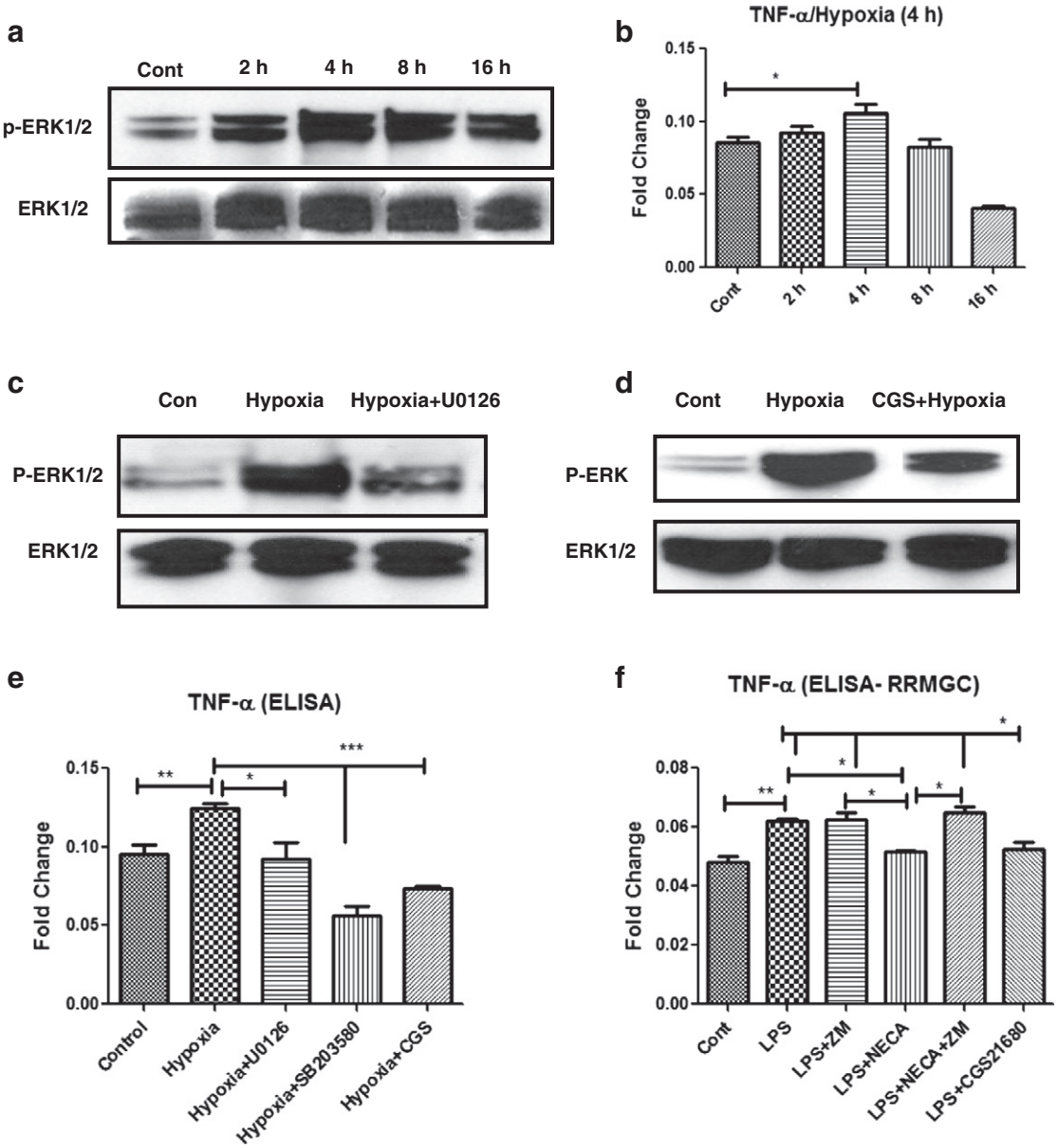

Fig. 7. Role of MAPKinase in the A_{2A}AR-mediated anti-inflammation: analysis of hypoxia- & LPS-induced TNF-α release in the retinal microglial cells. a) Time-dependent, hypoxia induced activation of ERK in the retinal microglial cells. Cells were treated with hypoxia for 2–16 h. Phosphorylated (p) ERK and its total protein in cell lysate were determined by Western analysis. b) TNF-α release was measured by ELISA in time-dependent hypoxia. c–d) Selected time point (4 h) study in retinal microglial cells for hypoxia-induced pERK activation and TNF-α release, and its inhibition with U0126 (MEK inhibitor). e–f) Retinal microglial cells were treated with CGS21680 (20 μM) for 4 h. p-ERK and TNF-α releases were determined by Western and ELISA analyses, respectively. Retinal microglial cells were treated with LPS (30 ng/ml), NECA (2 μM), ZM241385 (2 μM) and CGS21680 (20 μM). TNF-α release was determined by ELISA. Data shown are the mean ± SD (n = 4). *P < 0.01, **P < 0.001, ***P < 0.0001.

A_{2A}AR as a critical control point for TNF-α release in hypoxia- or LPS-treated retinal microglial cells.

3.7. A_{2A}AR signaling mediates the anti-inflammatory effect via interaction with hypoxia-activated MAPK pathway in retinal microglial cells

The role of MAPKinases in the hypoxia-induced TNF-α release was studied. Whether or not MAPKinases are modulated by the A_{2A}AR signaling was also examined here. As shown in Fig. 7a & c, ERK was hypoxia-activated, although following different time courses. As shown in Fig. 7e & f, CGS21680 inhibited hypoxia-induced ERK activation as well as TNF-α release. Collectively, these results suggest that the A_{2A}AR-signaling cross-talks with MAPK pathway to modulate hypoxia-induced TNF-α expression in retinal microglial cells.

4. Discussion

In traumatic optic neuropathy (TON), the injured optic nerve may lead to visual loss. Visual loss results from loss of retinal ganglion cells (RGC), a layer of cells that is continuous with the optic nerve. In TON, stress causes vascular and neuronal damage of the retina and activates microglial cells. It has been reported that the activated microglia increased the damage by secreting pro-inflammatory cytokines and cytotoxic molecules in response to oxidative stress (Kreutzberg, 1996). In the retinal microglial cells near RGC, extracellular adenosine is responsible for a mechanism of anti-inflammation mediated by adenosine receptor A_{2A} (A_{2A}AR) signaling (Ibrahim et al., 2011b). Adenosine, which is a ubiquitous purine neucleoside, has been reported to have many diverse functions in metabolic stresses such as hypoxia and inflammation. Adenosine works through its interaction with specific 7-

transmembrane receptor subtype. Evidence suggests that $A_{2A}AR$ has a potent anti-inflammatory function and is widely expressed on cells of immune system like microglia and macrophages (Milne and Palmer, 2011). The previous report of our group demonstrated that the activation of $A_{2A}AR$ blocks endotoxin induced inflammation in these cells (Liou et al., 2008). In this study, pharmacological experiments with $A_{2A}AR$ agonist CGS21680 is showing that $A_{2A}AR$ is the main candidate for mediating the adenosine effect on suppression of pro-inflammatory cytokines in activated microglial cells. The ability to offer protection of $A_{2A}AR$ and its agonists against inflammation and tissue injury in the kidney, liver, heart, lung, vasculature and brain have been demonstrated in a number of studies (Sheardown and Knutsen, 1996; Okusa et al., 1999; McPherson et al., 2001; Ohta and Sitkovsky, 2001; Fozard et al., 2002; Yang et al., 2005).

The present study showed that mouse retina with TON exhibits significantly increased oxidative stress, activated microglial cells, TNF-α release, and retinal cell death as compared with control retina and which may be effectively treated with $A_{2A}AR$ agonist. We have shown the data that treatment of hypoxia or LPS induced MAPK activation increased the accumulation of TNF-α in primary culture of rat retinal microglial cells, which further may lead to neurodegeneration. Treatment with $A_{2A}AR$ agonist in both the cases (in vitro and in vivo study) significantly blocked the MAPK activation and TNF-α release. To the best of our knowledge, this is the first report demonstrating that $A_{2A}AR$ agonist could be an effective therapeutics for TON. In in vivo study, we have shown increased cell death, cleaved caspase 3 activation and microglial marker Iba 1 expression in TON retina compared with normal retina. Pro-inflammatory cytokine (TNF-α and IL-6) production was significantly higher in the retina with TON. The $A_{2A}AR$ agonist administration significantly attenuated the expression of these inflammatory and cell death markers, and results show that $A_{2A}AR$ selective agonist evokes the anti-inflammatory activity in TON activated microglia cells via MAPKinase signaling. Using primary culture of microglial cells, we found that treatment with hypoxia or LPS significantly increased TNF-α level and activated ERK1/2 signaling. $A_{2A}AR$ agonist reduced ERK1/2 activation and TNF-α release in microglial cells (Fig. 7a–f). Recent study of our group has shown that $A_{2A}AR$ selective agonist inhibits Ras/C-Raf/MEK/ERK signaling in AGA-activated microglial cells (Ibrahim et al., 2011b).Stork and Schmitt (2002) has reported that on activation of Gs-coupled receptor to regulate cell function, cAMP increases to interact with the Ras/C-Raf/MEK/ERK signaling (Stork and Schmitt, 2002). The interaction between cAMP and Ras/Raf/MEK/ERK signaling may lead to activation or inhibition of ERK activity, which further regulates cytokine production in immune cells. Report suggests that $A_{2A}AR$ inhibits two major pro-inflammatory signaling pathways, the NFkB and the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways (McPherson et al., 2001). These pathways regulate inflammatory cytokines such as TNF-α and IL-6 production (Karin and Ben-Neriah, 2000; Ding et al., 2009). These studies further support our findings that $A_{2A}AR$ agonist suppresses pro-inflammatory cytokine release via different signaling pathways and in the case of TON, inhibition of MAPKinase pathway through $A_{2A}AR$ may regulate the increased TNF-α release in activated microglial cells.

We further investigated the role of $A_{2A}AR$ agonist on superoxide generation/ROS production in TON. Previous studies have shown that $A_{2A}AR$ inhibits superoxide/ROS production in leukocytes (Sullivan et al., 2001; Nadeem et al., 2009). However, our DHE and DCF data showed that $A_{2A}AR$ agonist significantly inhibits superoxide/ROS generation via inhibition of NADPH oxidase subunit gp91phox activation. NADPH oxidase and GFAP activation have been implicated in pathogenesis of several diseases including hypertension, stroke and diabetes, where increased generation of superoxide/ROS contributes in cell death (Roe et al., 2011; Bhatia et al., 2012; Tang et al., 2012). In agreement, another study has shown that $A_{2A}AR$ agonist CGS21680 inhibits NADPH oxidase activity via cAMP-PKA signaling pathway (Nadeem et al., 2009). Together, these results suggest that microglial NADPH oxidase could be the major source of ROS formation in TON and CGS21680 may attenuate its activation.

We also studied the role of $A_{2A}AR$ in TON by using $A_{2A}AR$-KO mice. Retinas from KO mice exhibit significantly more TNF-α release, ICAM-1 expression, activated microglial cells, and cell death compared to retinas from wild-type mice. Increased ICAM-1 expression has been implicated in increased retinal inflammation and permeability in diabetes (Joussen et al., 2004). Under stress conditions, the local levels of extracellular adenosine are elevated due to the increased need for energy supplied by ATP (Johnson et al., 1999) and the degradation of released ATP (Wurm et al., 2008). This increased adenosine can protect against excessive cellular damage in a negative feedback manner (Ralevic and Burnstock, 1998). However, the apparently impaired protection in mice with TON reflects that protection by endogenous adenosine is insufficient and CGS21680 treatment modulates this effect.

To prove the anti-inflammatory effect of $A_{2A}AR$, we used LPS to activate primary retinal microglial cells. In our earlier work, we have shown that LPS treatment activates microglial cells to stimulate inflammation and p38 mitogen activated protein kinase activation, which causes neuronal degeneration (El-Remessy et al., 2008). We pre-treated the microglial cells with an $A_{2A}AR$-selective agonist CGS21680, and the role of $A_{2A}AR$ in reducing TNF-α release was confirmed by non-selective agonist NECA and $A_{2A}AR$-selective antagonist ZM241385 followed by LPS treatment as shown in Fig. 7g. Our findings are supported by earlier work where CGS21680 reduces ischemic or excitotoxic hippocampal damage (Jones et al., 1998). In this study, we showed that in vivo efficacy of CGS21680 reduces retinal inflammation and cell death associated with TON. This evaluation of the in vivo effect of CGS21680 is important for the development of a receptor-based therapy for TON. Future challenges include the development of compounds with high and selective binding affinity to $A_{2A}AR$ approaches to deliver $A_{2A}AR$ agonists to retina, and the definition of pharmacologic strategies to safely use $A_{2A}AR$ agonists in patients with TON.

In summary, we may conclude that this present study yields preclinical evidence which demonstrates that $A_{2A}AR$ mediated signaling is a critical pathway for debilitating retinal cell death associated with TON (Fig. 8). Furthermore, the current study substantiates that the function

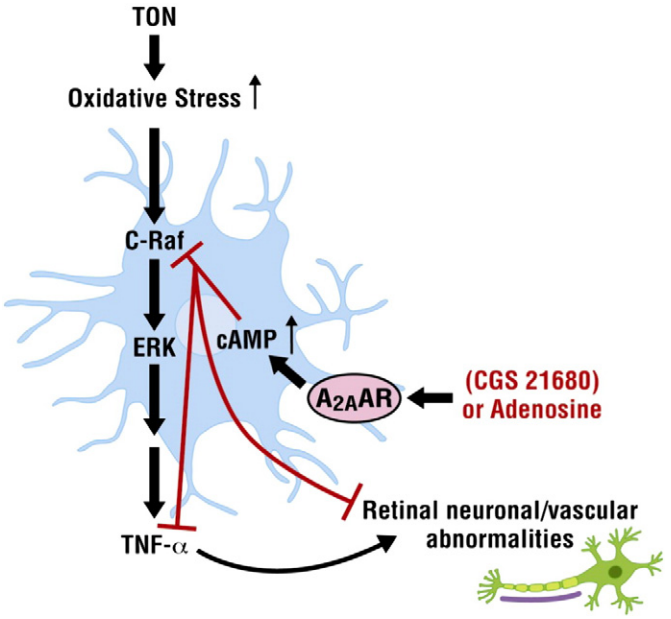

Fig. 8. Proposed pathway of oxidative stress and MAPKinase mediated TNF-α release, and A_{2A} adenosine receptor ($A_{2A}AR$) agonist mediated anti-inflammation in the retinal microglial cells during traumatic optic neuropathy.

of A_{2A}AR agonist to inhibit oxidative stress and MAPK-mediated inflammatory cytokine release in activated microglia represents a novel therapeutic approach to treat retinal degeneration associated with TON. Thus, A_{2A}AR has significant potential as a therapeutic target in TON.

Disclosure

This report is according to journal guidelines and ethical issues. All authors have no conflicts of interest.

Acknowledgments

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